Seasonal growth dynamics of two temperate New Zealand finfish, Australasian snapper (*Chrysophrys auratus*) and yellow-eyed mullet (*Aldrichetta forsteri*) – two strategies for the common success

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"7. *But ask now the beasts, and they shall teach thee; and the fowls of the air, and they shall tell thee:*

8. *Or Speak to the earth, and it shall teach thee:*

***and the fishes of the sea shall declare unto thee**."*

Job 12:7-8 King James Version

ABSTRACT

Proteins sourced from the sea make up a crucial part of global food production and considerably supports sustainability of human demographic growth. However, many fish stocks worldwide are overexploited or depleted and production enhancement is required. Here in New Zealand, certain finfish species are targeted to boost production to address the increase in market demand. One of them is Australasian snapper, *Chrysophrys auratus*, an iconic New Zealand species with a reputable history of exploitation and research whose growth potential is still not fully understood. When compared with kin species (e.g. *Sparus aurata* – North-East Atlantic /Mediterranean Sea and *Pagrus major* from North Pacific) it becomes obvious that a further increase in snapper production and exploitation in New Zealand waters should be possible.

The main objective of this thesis was to explore the maximum potential growth capacity and the underlying physiology of snapper, as well as yellow-eyed mullet (YEM), *Aldrichetta forsteri*, as a comparative model species. The objective was investigated at three levels: First, by measuring growth morphometrics, which includes measuring growth parameters, mass and length, and determining supplementary organosomatic indices from fish kept on an unrestricted diet that enabled maximum growth rates. Second, energy generation, utilisation and partitioning requirements in association with such growth performance could be addressed by investigating metabolic (i.e. resting and maximum metabolic rates and associated aerobic metabolic scope) and digestive (specific dynamic action, SDA – increase in metabolic rates due to feeding) capacities. The third addressed the question – what happens on the biochemical level in organs/tissues actively involved in maximum growth performance? This stage was investigated by determining concentrations of key tissue metabolites and activity of enzymes involved in pathways associated with energy production necessary for growth as well as those related to digestion. Finally, this thesis intended to describe characteristics of food limitation in the natural environment of the test species and what would be possible if anthropogenic feeding of wild stocks, as a relatively novel approach for stock enhancement and production expansion, would be introduced.

To address the question about maximum growth potential, tank array food unlimited experiments were set to investigate what would be the fastest growth rate that test species can achieve in the wild,

based on the assumption that food is limited in the natural habitat. Therefore, the experimental set-up was designed to mimic environmental conditions so that the obtained data could be compared with growth from wild populations. Fish were fed *ad libitum* for the period of one calendar year and monthly growth variables were measured, and on that basis, annual growth trajectories were constructed. During monthly measurements, fish were sacrificed for tissue and organ sampling required for biochemical profiling. This protocol also provided the opportunity to gather data to compute organosomatic and related indices as contributing information supplementing growth data in explaining growth profiles of the model species. Overall, snapper demonstrated an explicit seasonal growth trajectory where an intense growing season was associated with spring/summer and growth reduction/cessation with winter. YEM, on the other hand, exhibited constant annual growth where challenging winter conditions (e.g. low temperatures) had only minor effects on slowing growth down. In addition, this work demonstrated that one of the most important eco-physiological traits of teleost fish when food is abundant could be to ensure that energy stores are optimised. This appeared to be particularly important for YEM. In snapper, though, with increasing seasonal temperatures after winter, growth compensation was emphasised, but just before winter, growth in mass/length was marginalised and energy reserve deposition prioritised. These findings also closely agreed with the biochemical profile.

Metabolic parameters (resting and maximum metabolic rates, and SDA) were investigated at a set of temperatures (i.e. 13, 17 and 21°C), which represent the temperature range that test species experience in natural environments on a yearly basis. Therefore, these data could also be linked with the food unlimited trials. In addition, SDA was determined for a range of different rations to investigate the effects of meal size on SDA response. Temperature was a strong modulator of snapper SDA profile where most parameters measured responded with a clear pattern, whereas YEM had only SDA duration shortened when temperature increased from 17 to 21°C. Similarly, in terms of meal size, all aspects of snapper SDA were affected; still, differences between 0.5 and 1% BM (body mass) rations (only rations that YEM were successfully fed) were not detected for both species. Interspecific comparison revealed that temperature differently affected SDA of 2 species while meal size had more similar effects. Overall, characterisation of metabolic parameters demonstrated that snapper possesses an aerobic capacity that is generally inferior to that of YEM. This was also supported by the findings from the biochemical investigation. Besides, it was evident that temperature affects how snapper assimilate nutrients and partition energy between stores and somatic growth more than YEM.

Generally, snapper and YEM demonstrated that they possess two different growth strategies, where YEM on an annual basis prefer to grow unceasingly, which may allude that YEM could have superior growth to snapper. Nevertheless, the capacity of snapper to catch up what was lost during the cold season, once conditions for growth improved in spring/summer, showed that this may not be true. When snapper growth, in terms of muscle/fillet mass, was compared with YEM there was virtually no

difference between the two. This demonstrated that neither growth strategy can be regarded as advantageous but rather different. Besides, experimental data were found to be greater than what was found in the wild, suggesting that food may be a limiting factor for the test species to accomplish their maximum growth potential, either because of its seasonal patchiness and/or reduction in quality.

CHAPTER 1

General Introduction

1.1 World fisheries, and New Zealand's place on the global market – constraints and remedies

Our world is a dynamic place where unstoppable demographic expansion imposes a toll and cannot continue at today's pace. Indisputably one of the biggest demands is an adequate food supply and its worldwide distribution (FAO, 2016). Fish protein is placed high on the list to help satisfy this demand, currently serving over 17% of world protein requirements (Food and Agriculture Organization of the United Nations - FAO, 2016). This is also outlined in a statement from a previous FAO report (2014) on the state of world fisheries and aquaculture that reads: "People have never consumed so much fish or depended so greatly on the sector for their well-being as they do today". However, the current state of the fishery and aquaculture industry is debatable. Pauly and Zeller (2016, 2017) have fiercely attacked statistical analysis and future projections on global marine fisheries stated in the last two FAO (2014, 2016) reports, and emphasised that presented total annual commercial catch for the last 60 years, and specifically the period between 2009 and 2014 with average catch in excess of 90 million tonnes, is severely underestimated and consequently the projections of global decline in production are expected to be much steeper. On the other hand, prominent fishery biologist Ray Hilborn and his team are aligned with FAO, especially where the world organisation expressed the condition of global fishery and aquaculture as achieving new goals of 20 kg fish per capita in 2014 based on intense growth in the aquaculture sector and slight but steady improvements in fisheries management that resulted from rebuilding certain fish stocks (Hilborn and Hilborn, 2012; FAO 2014, 2016; Costello et al., 2016). However, the real focus should not be on opinion disputes but rather on the fact that across global fisheries, of which many are already fully exploited (58.1% of all assessed fish stocks, and only 10.5% are underfished, FAO 2016), implementation of effective management reforms are still required (Merino et al., 2012; Costello et al., 2016). In addition, investigation into alternative options (i.e. free ranging/ranching, advanced aquaculture) to enhance fisheries is welcomed more and more and is gaining increasing acknowledgement in recent times (Hallderson et al., 2012).

1.1.1 New Zealand is a conspicuous stone in the global fishery mosaic

Out of 28 leading fishing nations (encompassing over 80% of worldwide total fish catch) New Zealand is positioned 5th according to the Fisheries Management Index whereby management systems by species were characterised in regard to research, management, enforcement, socioeconomic attributes and stock status (Melnychuk et al., 2017). In addition, Marchal et al. (2016) stated that New Zealand, the EU, Iceland and Australia are amongst the most innovative world fishery nations due to introduction and implementation of novel management practices such as stakeholder involvement, legally-binding management targets, individual transferable quotas and discard bans. On top of that New Zealand is striving to raise the game to another level by advocating and strategically implementing an ecosystem approach to fishery management, 'provide for utilisation while ensuring sustainability', which surfaces from the New Zealand Fisheries Act 1996 (Cryer et al., 2016). The approach is based on practical measures regarding issues such as fish bycatch; incidental captures of protected species; changes to marine biodiversity; benthic effects caused by dredge gear and bottom trawl; and the protection of habitats of particular significance for fisheries management (Cryer et al. 2016). However, despite objectively well managed systems and significant resource input into further expansion of the sector there are still many uncertainties and lots of space for improvement and redevelopment since many important New Zealand fisheries are over or maximally exploited (Ministry for Primary Industries, 2017). More specifically, 27 of 160 (i.e. 16.9%) assessed stocks in 2015–16 were depleted or overfished and need to be systematically rebuilt. A whole suite of proposed approaches to increase productivity and enhance sustainability of fish production in New Zealand are on the horizon, such as directed (i.e. beyond single) fish stock management (Bess, 2005; Cryer et al., 2016), innovative aquaculture (Alfaro et al., 2014), stock enhancement (Handleya et al., 2016), fish farming (Symonds et al., 2014), free ranging, etc.

But the question should not only revolve around what the human sector can do, but extend to what can the fish do? Can fish perform better on the global food production stage if they are treated in a different way without compromising their wellbeing? Ultimately, they are highly efficient protein manufacturing biological entities. Therefore, finding an optimum biological "comfort" zone for growth is on the list of priorities that will underpin all other investigations into fisheries productivity in the future (Cacho et al., 1991; Björnsson et al., 2001; Buckley et al., 2004; Shelton et al., 2006; Benstead et al., 2014; Khan et al., 2014; Svedäng and Hornborg, 2014; Le Pape and Bonhommeau, 2015; McCann et al., 2016; van Poorten and Walteres, 2016).

1.2 Fish growth as a fundament of fishery industry

Since growth is a phenomenon on which fisheries, or any industry that involves exploitation of animal resources, is based a detailed understanding of its underlying processes is paramount for any further enhancements (Higgins et al., 2015). From the exploration of compensatory growth (an augmented growth when more favourable conditions are reinstated following impaired growth caused by food deprivation) it is evident that animals sometimes grow at rates below their maximal genetic potential (Ali et al., 2003).

Genetic tools (e.g. selective breeding) are an effective way to tap into this concealed potential that is locked into the genetic makeup (De-Santis and Jerry, 2007). However, only in recent times have genetic technologies through systematic breeding programmes started to become standard in aquaculture production practices (e.g. Atlantic salmon, Gjoen and Bentsen, 1997; and genetically improved farmed tilapia (GIFT) tilapia, Circa et al., 1995), and are still undeveloped compared with other research areas (De-Santis and Jerry, 2007). Amongst other reasons for the challenging status, there is the lack of understanding of genetic traits related to economic (productivity and profitability) requirements, such as growth rates, which are a key attribute of interest in selection programmes that currently encompasses the finfish sector (Jerry et al., 2001; De-Santis and Jerry, 2007). In New Zealand the genetic approach has become more conspicuous and is gradually gaining momentum regarding aquaculture and marine farming (see MacAvoy et al., 2008; Camara and Symonds, 2014).

Boosting wild stock growth via anthropogenic feeding is an area that has been explored in recent times that yielded encouraging results (Björnsson, 2002, 2011). This pathway started to become more prominent in the late 90s and early 2000s when the idea was tested on Atlantic cod (*Gadus morhua*) by implementation of a classical Pavlovian conditioning using supplementary feeding of wild stocks (Björnsson et al., 1999; Björnsson et al., 2001). The outcomes suggested a number of desirable advantages such as increased growth rate of target species, eased rate of predation on important taxa and reduced costs compared to conventional fishing practices. Moreover, based on a large-scale comparative study, Hallderson et al. (2012) showed that cod ranching was more profitable than conventional fishing and full-cycle farming. Relevant New Zealand institutions (e.g. Crown Research Institute Plant & Food Research – PFR, amongst others) considered the idea to be trialled in New Zealand waters since some species (i.e. snapper *Chrysophrys auratus*) were evaluated as a potential candidate (Coubrough et al., 2004).

In addition, fish farming and advanced aquaculture, which are also in the stage of intense exploration in New Zealand, similar to selective breeding and free ranging, require a suitable growth

profile for any advancement towards the introduction of new species into commercial-scale production (Alfaro et al., 2014; Symonds et al., 2014;). On top of that, the recovery pathway of depleted New Zealand inshore fisheries requires further understanding of biological properties of affected populations since there is still much unknown about our native species (McCarthy et al., 2014).

It appears that all the aforementioned areas of production or research, which already are or are steadily becoming important and appreciated in addressing industry growth in New Zealand, need specific knowledge on which further research can be based. One of the cornerstones of that fundament is a detailed knowledge of a potential candidate's biological growth profile (Symonds et al., 2014). Typically, a growth profile is characterised by evaluating growth performance traits such as growth rates, preferably explored for all seasons to account for seasonal variations, condition and body indices, growth biochemical correlates (i.e. aerobic, glycolytic and digestive enzymes), growth related low molecular mass tissue compounds (i.e. tissue lactate and glucose) and energy store metabolites (i.e. tissue glycogen) (Jobling, 2008). Growth performance is influenced by a complex suite of exogenous (environmental) and endogenous (genetics, age, sex) factors which has been challenging to quantify in population studies, namely across stocks (Brander, 1995). Therefore, laboratory or tank array studies under a controlled environment as an alternative route has been successfully carried out in many instances (Björnsson and Steinarsson, 2002).

Taking all of these knowledge requirements and industry challenges into consideration it makes a solid argument to start addressing these matters with exploration of base-line growth potentials of important and prospective finfish species in a controlled environment to build an understanding of their growth profiles. Furthermore, in order to tap into the maximum potential growth rates of these species that their current genetic make-up will allow, long time-scale (i.e. one calendar year) growth experiments should be carried out, accompanied by food-unlimited feeding conditions to support maximisation of growth performance with an all-encompassing monitoring programme that includes determination of growth rates, condition and body indices, growth biochemical correlates, growth related tissue metabolites and energy stores and their annual dynamic. This scenario describes a compendious outline of the main theme of this thesis.

1.3 Growth in fishes, definition and regulation – overview

Growth is a singularity that has been given many definitions in the past (e.g. Pauly, 1979; Jobling, 1994; Boeuf and Payan, 2001; Enberg et al., 2008; Higgins et al., 2015). Nevertheless, most of them can be summarised into two relatively simple views. First, based on the von Bertalanffy's quantitative theory of organic growth published in 1938 (van Poortena and Walters, 2016), growth is explained as a net product of two contrasting processes in an organism, one that promotes an increase (anabolism) and the other a decrease of body mass (catabolism) (Pauly, 1979). And second, based on energy partitioning theory, growth is defined as a consequence derived from a difference between what comes in the body and what vacates it (Brett, 1979; Cho et al., 1982; Weatherley and Gill, 1987; Elliott, 1994; Jobling 1994, 1997). This definition can be presented mathematically in the following way:

$$\frac{\delta M}{\delta t} = pR - C$$

where $\delta M/\delta t$ is a mass change in unit time (representing somatic and reproductive growth); p is a coefficient representing the energy content in food or nutrient availability; R is meal size and C denotes losses through catabolic activity. According to this view growth's phenotypic expression is predominantly determined by quality and quantity of ingested food. However, growth trajectory is shaped by a large array of internal factors, which are important with respect to species (i.e. genetic makeup with its regulating system and hormonal control), and external factors, which are essential from the ecological aspect of animal existence (i.e. environmental conditions – dietary component/food availability and physical and chemical properties of surroundings) (Dutta, 1994; Moriyama et al., 2000; Boeufa and Payanb, 2001).

When growth is considered within the subphylum of vertebrata, fish seem to have an exclusive position, as apart from a few exceptions, fish exhibit indeterminate growth (Moyle and Cech, 2004). In other words, fish show an increase in size and mass even after sexual maturation, which is continuous for the rest of their lifetime, providing that the food supply is sufficient and there is no shortage of space (Mok, 2008). Growth itself is mostly expressed as an increase in mass of white muscle, which accounts for up to 80% of fish mass growth (Weatherley and Gill, 1985; Mommsen, 2001). Around two-thirds of metabolic activity related to protein synthesis in muscles belongs to the production of myofibrillar proteins that are mainly taken up by myosin heavy chains (Mommsen, 2001). The presence of transcripts for the myosin heavy chains throughout a fish lifespan represents another unique feature of fish biology that appears to be linked to indeterminate growth (Gauvry and Fauconneau, 1996). Although fish tend to grow constantly, the growth is not linear, and it is composed of the combined effects of hypertrophy (increased size of cells) and hyperplasia (increased number of

cells) that both contribute to growth throughout their lifespan, which differentiates them from higher vertebrates such as mammls (Mommsen, 2001). However, the contribution of hyperplasia may decrease progressively with age (Alfei et al., 1994).

1.3.1 Genetic control of fish growth – overview of major pathways

Mainstream research in the area of genes involved in regulating fish growth has been governed by the fact that the majority of fish growth is accounted for by muscle mass increase (Ulloa et al., 2011). Numerous genes linked to somatogenesis (i.e. muscular growth) that previously had been identified (mapped and sequenced) in mammals have been repeatedly found in fish (Lo Pestri et al., 2009). The following is a summary with a description of the main genes/ gene groups that have been discovered to play a vital role in fish muscle growth:

The fish gene group that has been the most investigated in relation to somatogenesis is the one associated with the somatotropic axis and transforming growth factor (Ulloa et al., 2011). Those genes are major regulators of both endocrine and autocrine physiological and growth related metabolic processes, and hence play a fundamental role in skeletal muscle growth (Moriyama et al., 2000; De-Santis and Jerry, 2007). Another controlling factor, myostatin or differentiation factor 8, is responsible for down regulation of fish muscle growth and it has been located in many fish tissues including kidneys, gonads and gills (Ulloa et al., 2011). Myogenic regulatory factors, on the other hand, are a group of transcriptional factors associated with myogenesis (skeletal muscle formation) that are specifically expressed in the skeletal muscles of vertebrates (Atchley et al., 1994). When glycoprotein follistatin was first discovered in higher vertebrates, it was understood that its primary function was inhibition of follicle-stimulating hormone secretion (Macqueen and Johnston, 2008). Subsequently it has been identified as a regulator of myogenesis and an inhibitor of growth factor in fish (Macqueen and Johnston, 2008). Another important growth gene, calpain-calpastatin (CAST) system, has also been discovered in fish. Its role has found to be in regulating protein turnover, myoblast (precursor embryonic cells to be developed to myocytes/muscle cells) differentiation and cell cycle progression (Goll et al., 2003). Salem at al. (2005) observed the upregulation of mRNA transcripts for the CAST gene system in a starving rainbow trout. And finally, genes representing the ubiquitin-proteosome proteolytic pathway are controlled by nutritional status and seem to be responsible for skeletal muscle protein degradation (Ulloa et al., 2011).

1.3.2 Hormonal control of fish growth

The main hormonal system that promotes growth in fish is the growth hormone (GH)-insulin-like growth factor (IGF) system (Glass, 2005; Wood et al., 2005; Velloso, 2008; Fuentes et al., 2013). The system is established through the somatotropic axis, which is formed of growth hormone-releasing hormone (GHRH), growth hormone (GH), growth hormone receptor (GHR), insulin-like growth factors (IGF-I and II) with their specific carrier proteins and receptors, and insulin (Ulloa et al., 2011). GH is a peptide hormone formed by somatotropic cells located in the anterior pituitary with regulatory support of somatic growth through direct and indirect pathways (Mommsen, 2001; Rius-Francino et al., 2011; Fuentes et al., 2013). GH has a direct influence on the CNS, which may be demonstrated as an alteration in appetite, aggressive behaviour and overall activity patterns. Furthermore, the direct impact on protein synthesis is by activation of the muscle GH receptors or by promoting production and release of local IGF-I (Mommsen, 2001; Fuentes et al., 2013). Indirectly, GH supports growth by promoting intestinal growth, increasing amino-acid uptake through the intestinal wall and Na/K ATP-ase and glucose transport activity (Reshkin et al., 1989; Farmanfarmaian and Sun, 1999). Besides, GH diverts amino acids from oxidation into growth by inhibiting lipogenesis and promoting lipolysis, which both contribute to an increase in free fatty acids as a more desirable oxidative substrate (Mommsen, 2001). IGF is a mitogenic (i.e. promotes/triggers mitosis) polypeptide from the insulin superfamily and it functions in cell transformation, inhibition of apoptosis (controlled death of cells) and mitogenesis, mediated through activation of its receptors (Glass, 2003, 2005). Additional roles of IGF that have been found in fish also include tissue regeneration, osmoregulatory acclimation and reproductive development (Woods et al., 2005). Insulin is a 51 amino-acid hormone produced by the pancreatic B cells with a high plasma concentration in adult fish relative to mammals (Urbinati et al., 1994). Its binding activity per gram of tissue is particularly high in white muscle compared with liver and it can be 24–27 times higher, as has been demonstrated for brown trout and carp (Parrizas et al., 1995). Insulin in fish may not have similar functions as it does in mammals due to the unclear existence of an insulin-sensitive glucose transporter (Wright et al., 1998). Therefore, it acts more as a general mitogen and growth promoter through supporting amino-acid uptake and muscle protein synthesis, and thus it appears to have an important role in somatic growth in fish (Mommsen, 2001; Castillo et al., 2004).

1.4 Environmental factors influencing fish growth

Relating growth of fish with environmental factors has been a very prolific area of research that contributes to understanding how vital an organism's dependence on its ecological surroundings is (Jobling, 2008). Boeufa and Payan (2001) stressed the importance of understanding the role of external, namely environmental or ecological factors, on growth of teleost fishes as being crucial since growth is in most cases directly under their control. They further systematised those ecological parameters into an array of determining (i.e. those that are detected via fish sensory systems such as temperature, salinity and light) and limiting factors (i.e. those that affect fish growth performance when optimal threshold of an environmental variable, such as level of dissolve oxygen, has been breached). The majority of studies have dealt with the impacts of one or a few factors on growth performance. This is because in many instances it is not easy to distinguish the effects of all key environmental variables on growth simultaneously since the level of collinearity and interdependence amongst them is very high; for example, the natural seasonal variability in sea-water temperature, photoperiod, food availability and dissolve oxygen concentration have a propensity to follow a similar cycling rhythm (Jobling, 2008).

Since the effects of food availability and sea-water temperature are variables of particular interest to this thesis they will be elaborated in detail in Section 1.5 and later discussed in relation to the experimental findings. Here is a general overview of other environmental factors which are also considered to be highly influential upon fish growth:

Salinity – Fish are adapted to their saline environment, therefore they are either stenohaline (tolerating a narrow envelope of salinity gradient) or euryhaline (tolerating wider or even extreme ranges of salinity such as the case of milkfish, *Chanos chanos*, enduring a range of 0–55 ppt as reported in Swanson (1998) (Dutta, 1994; Overton et al., 2008). The majority of fish species are particularly sensitive to change in salinity in the early stage of growth and development, which includes egg fertilization and incubation, early embryogenesis, swimbladder inflation and larval growth (Boeuf and Payan, 2001). In later growth stages fish exposed to salinity levels that are outside of their optimum tolerance range will often exhibit compromised growth, which appears to be due to an energetically costly increase of Na/K–ATPase, the activity of which is necessary for body osmoregulation (Sampaio and Bianchini, 2002). Furthermore, many studies investigating growth performance for possible commercial exploitation observed that the best growth rates were found in fish kept at intermediate salinities (8–20 ppt) rather than in full-strength sea-water (~35 ppt) (Boeuf and Payan, 2001; Imsland et al., 2008).

Light – There are three aspects of light: photoperiod, light intensity or quantity and light colour spectrum or quality and their impacts on growth can be separately evaluated (Boeuf and Lebail, 1999; Puvanendran and Brown, 2002). Fish growth can often be enhanced by manipulations of these factors since they positively affect both behaviour and physiological responses in fish (Boeuf and Lebail, 1999; Puvanendran and Brown, 2002; Stuart and Drawbridge, 2011; Prayogo et al., 2012; Honryo et al., 2013; Wang et al., 2015).

Photoperiod – Photoperiod has been found to have a species-specific effect on growth of fresh water fish taxa (i.e. brown trout, *Salmo trutta*, prefers short but cyprinids long day photoperiod). However, for most marine species including some salmonids (i.e. *Salmo salar*) long daylength stimulates growth (Dutta, 1994; Boeuf and Lebail, 1999). Therefore, it is necessary to discover an appropriate photoperiod for a species for growth enhancement, as it has been demonstrated to promote food intake, nutrient assimilation and in addition it reduces density dependent stress (Fielder et al., 2002; Trippel and Neil, 2002; Howell et al., 2003; Imsland et al., 2006; Ballagh et al., 2008; Martínez-Cárdenas and Purser, 2011; Prayogo et al., 2012).

Light intensity or quantity – light intensity is especially crucial for larval development where a minimal light intensity threshold is required otherwise larval mortality rate can be markedly increased (Boeuf and Lebail, 1999). However, the range of minimum requirements may vary substantially amongst species, since species occupying a demersal environment with elevated turbidity (i.e. flounder) may prefer lower ranges of intensity for optimum growth (Alvarez-Verde, 2015). Nevertheless, a minimum level of light intensity is paramount for larvae and juveniles and to a certain extent adult foraging behaviour as it permits food visualisation, amplifies food intake and assimilation, supports immune system development, increases enzymatic activity and consequently promotes higher growth rates (Trippel and Neil, 2003; Ashley, 2007; Stuart and Drawbridge, 2011; Honryo et al., 2013; Wang et al., 2015). But on the other hand, light that is too intense often causes a stress response and in extreme cases increased fish mortality (Boeuf & Le Baile, 1999).

Light spectrum or quality – Intrinsic optical sea-water properties not only affect a change of light intensity with increasing depth but also its spectral profile. Therefore, it is also expected that colour may have an effect on fish growth performance (Takahashi et al., 2016). Although this area of research is still relatively undeveloped, studies are emerging showing how different wavelengths influence food intake and increased growth rates in some fish species (e.g. Papoutsoglou et al., 2000; Karakatsouli et al., 2007; Luchiari and Pirhonen, 2008). Furthermore, recent studies have demonstrated that not only specific wavelengths were linked to the increased growth, but also underlying mechanisms through which the effects may be achieved have been proposed. In Takahashi et al. (2016) it was demonstrated that the stimulating effects of background green light on barfin

flounder (*Verasper moseri*), apart from having positive effect on somatic growth, was also evident as a response of the endocrine system associated with the growth increase. Furthermore, in some species there may be an ontogenetic effect with wavelength sensitivity changing with growth, for example, as has been demonstrated for *C. auratus* (Robinson et al., 2017).

Oxygen – Oxygen is vital for all animals as a base for energy turnover, and in some instances is a more influential limiting factor of growth than food supply (Fry, 1971; Brett, 1979). Hypoxia or low concentrations of dissolved oxygen (DO, < 1.4 mL O_2 L^{-1}; < 60 μmol O_2 L^{-1}; < 2 mg O_2 L^{-1}; < 30% saturation) causes physiological transitions that may affect many aspects of fish biology including growth capacity and performance even if food supply is adequate (Ekau et al., 2010). However, this applies only if the DO level drops in the environment that a certain fish is adapted to or in high stock density scenarios of intensive aquaculture. Hypoxic DO concentrations are not the same for all fish (i.e. species-specific) as some are adapted to lower and some to higher DO concentrations (Jobling, 1994). An important phase in fish development when DO should be maintained at optimum levels is immediately after hatching when the demand for oxygen is markedly increased (Miyashita et al., 1999; Dong et al., 2011). During the juvenile stage, increasing DO concentration was often followed with increased food intake and growth rates (Tran-Duy et al., 2008, Wexler et al., 2011); and an opposite effect has been observed and frequently studied (e.g. see Jobling, 1994; Chabot and Dutil, 1999; Pichavant et al., 2001; Ekau et al., 2010). However, according to Pauly (1979, 1981) tissue metabolic oxygen demand is limited by its supply which is carried out via gills. Gill surface area determines the rate of supply, which becomes progressively more limiting with age since it is allometrically related to body mass, therefore bigger fish have lower oxygen uptake relative to their mass than smaller fish, which in turn results in stronger growth in younger than older fish (Pauly, 1979; Tran-Duy et al., 2008). However, this suggestion has been disputed in some other studies (e.g. Blier et al., 1997).

1.5 Fish growth in relation to food availability and ambient temperature

Food availability and ambient temperature of fish surroundings have been highlighted by many authors as the two most influential environmental variables to affect growth and its parameters (e.g. Meekan and Fortier, 1996; Björnsson et al., 2001; Baumann et al., 2003; Rindorf et al., 2008; Arnason et al., 2009; Auer et al., 2015; van Poorten and Walters, 2016). Therefore, interactions of effects on growth, due to their high correlation, were often investigated simultaneously, especially in instances of field experiments where manipulating for one to disentangle levels of impact of the other is challenging (Arnason et al., 2009). However, in a controlled laboratory environment while keeping

one variable constant it is possible to expose the precise effect of the other factor on growth performance (Arnason et al., 2009). In addition, Jobling (1994) stated that since temperature affects growth via its regulative impact on ingestion and metabolism it is important to distinguish between effects of temperature as such and the combined or interactive effects of temperature and food supply. But that does not mean that the underlying mechanisms should be portrayed and discussed by a single variable only, especially when temperature effects are the primary objective, since feeding conditions will certainly have a strong leverage on growth outcomes (Jobling, 1997). The most commonly reported link between temperature and available food supply on fish growth is that temperature has both a direct impact on growth via regulation of energy metabolism and rate of food assimilation (Krohn et al., 1997), and indirect effects through modifying prey availability, feeding behaviour and reproductive physiology (Schwalme and Chouinard, 1999). However, some authors argued that it is difficult to discern whether temperature operates directly on growth parameters via affecting metabolic rates or the main effect of temperature lies in indirect regulation of food abundance (Rindorf et al., 2008).

1.5.1 Fish growth in relation with food availability

Fish use the same survival strategy as other animals to forage, consume and convert a fraction of ingested organic substance into their own biomass, which depends on the quality and quantity of available food and the environmental conditions they are adapted to (Jobling, 2008; van Poorten and Walters, 2016). In terms of quality or type of food that fish are accustomed to, out of almost 34,000 known extant fish species (Froese and Pauly, 2018 – www.fishbase.org) for at least 600 the diet is known and accordingly fish can be classified as carnivores (85%), herbivores (6%), omnivores (3%) and detritivores (2%, that includes scavengers and parasites) (Dutta, 1994). From the early stages of fish life histories food appears to play a crucial role in their survival rates (Clemmensen, 1993). When there is an occurrence of poor larval year class strength it is hypothesized that two scenarios – lack of food or a mismatch (i.e. hatching time does not match with the timing of seasonal primary production bloom) are the most likely candidates to explain the phenomenon (Hunter, 1976; Lasker, 1987). However, after larval metamorphosis, there is no agreement in views when it comes to food limitation in the coastal and estuarine habitat where many juvenile fish species have an opportunity to enhance their fitness by optimising growth due to better feeding conditions, as the food limitation hypothesis states (Beck et al., 2001). Even though it has been commonly declared and accepted that food resources are a limiting factor in the capacity of nursery areas (van der Veer and Witte, 1993; Nash and Geffen, 2000; Craig et al., 2007; Nash et al., 2007), many studies have emerged demonstrating no effect of food limitation in nursery habitats, since juvenile growth was not affected by trophic conditions in the surveyed locations (Curran and Able, 2002; Ross, 2003; Hampel et al., 2005; Diaz et

al., 2011; Selleslagh et al., 2012). Therefore, it has been proposed that the carrying capacity of the habitats has not been exceeded (Rogers, 1994; Shi et al., 1997, van der Veer et al., 2000; Vinagre and Cabral 2008). However, a twist to the controversy was posed by Le Pape and Bonhommeau (2015) where they demonstrated that the apparent lack of growth limitation observed for juvenile fish in nursery areas was related to an observational bias, since only surviving fish were observed while size-selective mortality linked to food limitation was responsible for the nature of the findings. In the end Le Pape and Bonhommeau's (2015) explanation could not account for all cases in the debate, therefore it appears that the core eco-physiological principles in this matter are still cryptic and require further investigations (Juanes, 2007; Le Pape and Bonhommeau 2015).

Fish maintenance or resting metabolic rates (i.e. baseline energetic requirements of maintaining the physiological processes essential to sustain life; Fry, 1971) are a physiological trait that show plasticity or a level of intraspecific variation within populations even after accounting for effects that are due to differences in sex, age and mass (Kvist and Lindström 2001; Labocha et al., 2004; Steyermark et al., 2005). This was found to be because of individual differences in fish condition based on their life history account and prey availability (Biro and Stamps, 2010; Burton et al., 2011). Besides, it is more evident (see Guppy and Withers, 1999; McKechnie, 2008; McCue, 2010) that food availability may cause a shift in resting metabolic rates in a relatively short period of time (i.e. several days). In relation to this observation Auer et al. (2015) working on brown trout (*Salmo trutta*) demonstrated that flexibility in energy metabolism was a crucial attribute to optimise growth performance in conditions where food availability was variable. Individuals in their study that were able to increase resting metabolic rates more, when food was plentiful, exhibited higher growth rates, but when food conditions were reversed the best growth results were observed with fish that managed to decrease their resting rates the most.

Compensatory growth – Another aspect of growth physiology where food availability plays a vital function is in compensatory or "catch up" growth since many animals including fish demonstrate more rapid growth rates when they are in recovery after a period of food deprivation than conspecifics kept under undisturbed food supply (Wilson and Osbourn, 1960; Jobling, 1994). This may have important applications for aquaculture or other commercial purposes because fish experiencing compensation in growth rates, due to variable feeding conditions, in many instances achieve the same size-at-age as the same age fish under constant favourable conditions (Ali et al., 2003). However, compensatory growth was found not to have the same outcome in all cases but rather that results can vary according to four possible scenarios: the rarest is over-compensation where fish submitted to variation in food supply exhibit better growth performance at the end of trials than the control group kept under the balanced diet; no compensation occurs if fish after returning to a favourable diet regime exhibit growth rates typical of the size reached at the end of the food deficiency period; partial compensation is characterised by greater growth rates in food deprived group after re-establishment of diet as the

rates for the control group but the final size is below the size of the control group; and the most frequent response is a full compensation where both treatment and control groups are at the same size at the end of the trials (Ali et al., 2003).

Food availability and back calculation length at age determination – The number of fish scales in general do not change throughout the life, which implies that scales grow concurrently with the body in relatively constant proportion (Jobling, 2008). This relationship is of particular interest for fish from temperate regions as seasonal effects on food availability (i.e. the difference in food availability between growing time in spring and summer and impaired growth during the winter is substantial) is reflected in growth of body and simultaneously leaves distinguishable circular marks on scales that correspond with periods of intense growth over spring and summer and a period of slow or no growth over the winter (Casselman, 1990; Devries and Frie, 1996; Campana, 2001). Slow winter growth is characterised by smaller distances between circular marks (circuli) on scales which form recognisable ring like structures termed annuli (Zale et al., 2012). Therefore annuli, reflecting slow winter growth, can be used for fish ageing and for back calculating length at age which is a tool occasionally utilised in stock assessments and construction of growth curves of wild fish populations (Jobling, 2008; Zale et al., 2012).

Food availability and biochemical correlates of fish growth – The amount of food available for growth is also reflected in the biochemical organisation of body tissues as it corresponds with changes in growth rates and fish condition (Jobling, 2008). More specifically, certain enzymatic groups change their activity in accordance with nutritional status of an organism; in particular, anaerobic glycolytic enzymes (i.e. phosphofructokinase, pyruvate kinase and lactate dehydrogenase) in white muscle exhibit the most rapid activity decline as a consequence of fasting and vice versa, while aerobic mitochondrial enzymes (i.e. cytochrome *c* oxidase and citrate synthetase) appear not to be as strongly affected (Couture et al., 1998; Dutil et al., 1998). Therefore, monitoring activity of highly sensitive glycolytic enzymes in fish white muscle due to their observable sensitivity to food intake has become another practical tool in physiology as indicators of condition and growth (Couture et al., 1998; Dutil et al., 1998).

1.5.2 Fish growth in relation to environmental temperature

The surrounding temperature is a most important life governing agent for ectothermic organisms, and particularly aquatic ones since their growth traits are a reflection of feeding behaviour and metabolic activity, which are affected by temperature (Jobling, 2008; Pörtner and Farrell, 2008). Since most fish do not possess a thermoregulatory system, by definition they are thermoconformers, which

means that their body temperature oscillates in close proximity to ambient water due to its high specific heat capacity (Brill, 1994). However, since fish are mobile animals they are potentially able to choose more favourable thermal ambient locations, which essentially makes them behavioural thermoregulators (Jobling, 2008). When fish have the opportunity to select between sets of temperature or a position within a thermal gradient, as many experimental investigations demonstrated, fish settle at temperatures that highly coincide with those temperatures that were observed to support the highest growth performance (Magnuson et al., 1979; Jobling, 1981a; Kellogg and Gift, 1983; Jorgensen et al., 2012). The selected temperature will always sit within the boundaries of their thermal window (i.e. seasonal temperature fluctuation for the habitat) that can be considered as the thermal niche or thermal range that a species or life phase is adapted to (Pörtner, 2010). Eurythermal species that are equipped with an efficient oxygen supply system are able to cover a broader geographical/latitudinal extent (i.e. have higher thermal window), while cold stenothermal (i.e. narrow thermal window) fish are characterised by a low level of energy turnover as a trade-off ensuring a high level of growth efficiency (Heilmayer et al., 2004; Pörtner et al., 2005; Peck et al., 2009), since in the thermal envelope where an organism exhibits minimal cellular energy turnover growth will be maximised (Mark et al., 2005).

To distinguish the effects of temperature per se from combined or interaction effects of temperature and limited food supply on growth performance, food supply (or ingested food) should be controlled or kept constant (Jobling, 1994; Arnason et al., 2009). The most plausible approach that yielded tangible results in the past was to feed fish to satiation (i.e. *ad libitum* diet regime) according to their requirements as set by their internal (age and sex) and experimental conditions (temperature, photoperiod, etc.) (Björnsson et al., 2001, 2007; Arnason et al., 2009). Under this condition food intake tends to increase with temperature until the highest level of food consumption is reached close to the upper thermal limit for that fish (and its age) followed by rapid decline as temperature approaches its highest tolerable level (Fig. 1.1, Jobling, 1994). By the aid of growth rates plotted as a function of the same temperature range it becomes apparent that the peak growth performance or optimum temperature for growth is achieved at some intermediate temperature, which is not the same but rather to some extent lower than for the maximum food ingestion rate (Fig. 1.1, Jobling, 1994, 1997). Even though the support for this relationship has not been found across all research concerning fish growth and temperature (Larsson and Berglund, 1998; Forseth et al., 2001), the relationship has been observed in many studies and for many species (Brett, 1971; 1979; Cox and Coutant, 1981; Wurtsbaugh and Cech, 1983; Xiao-Jun and Ruyung, 1992). In addition, based on these two rates (growth and ingestion) the temperature impact on the efficiency level that food is converted into fish body mass, or conversion efficiency, can also be investigated (Jobling, 1997). Figure 1.2 indicates that the highest conversion efficiency is observed at the lowest temperature of three estimated parameters and sits approximately in the middle of the thermal tolerance window (Jobling, 1997). Findings in studies on behavioural thermoregulation suggest that preferred (selected) temperatures in most

instances do match the temperature that supports optimum growth, and in addition that there is an ontogenetic shift in thermal optimum with increased age and size of fish (Coutant and Cox, 1976; Hogendoorn et al., 1983; Keast, 1985).

Determination of optimal temperature for fish growth is an important area of research in applied physiology and aquaculture (e.g. Björnsson et al., 2001, 2007; Imsland et al., 2005; Khan et al., 2014). In laboratory conditions a pattern has been repeatedly observed describing a decrease in optimal temperature for growth with increased in fish size (e.g. Atlantic cod, *G. morhua* – Pedersen and Jobling, 1989; halibut, *Hippoglossus hippoglossus* – Björnsson and Tryggvadóttir, 1996; common wolfish, *Anarhichas lupus* – McCarthy et al., 1998; turbot, *Scophthalmus maximus* – Imsland et al., 1996; plaice, *Pleuronectes platessa* – Fonds et al., 1992). The same trend has also been observed for feed conversion efficiency (FCR), therefore it has been suggested that temperatures for both parameters (growth and FCR) are under ontogenetic influence due to a very systematic reduction in optimum temperatures for growth with increased mass observed for Atlantic cod (Fig. 1.3; Björnsson et al., 2001, 2007; Imsland et al., 2005). However, wild cod are typically found to congregate at a much lower temperature than temperatures determined under experimental conditions, which is assumed due to the difference in food supply – unlimited in those studies versus much lower levels in wild conditions (Björnsson et al., 2001). Furthermore, Imsland et al. (2005) demonstrated that FCR was the greatest for the group of juvenile Atlantic cod that was in temperature-step treatment (i.e. mimicking temperature condition in waters of wild fish habitats by dropping temperature for three degrees from 16 to 13 and 10°C for three consecutive months) but with a lower feed intake than cod reared at constant temperatures. This further corroborates the ontogenetic change in optimum temperature for growth in juvenile Atlantic cod (Imsland et al., 2005). Since growth experiments investigating temperature effects on growth performance are generally time and resource consuming, alternative approaches started utilising aforementioned thermoregulatory behaviour (temperature preference methods) of fish to predict the optimum temperature for growth and conversion efficiency (Khan et al., 2015).

As mentioned earlier, a rise in temperature is followed by an increased demand for oxygen, thus increased metabolic rates require more dynamic work of the respiratory and circulatory systems under progressively unfavourable decreased oxygen solubility (Jobling, 1997; Pörtner, 2010). However, it has also been observed that fish fed to satiation at a higher temperature, close to their upper thermal limit, had a lower oxygen demand relative to the incidents when they were under the same *ad libitum* feeding regime at a temperature a few (i.e. 3) degrees below. The reason for this discrepancy lay in the fact that fish at higher temperatures have reduced appetite, which appears to mirror the constraints of the oxygen delivery system to satisfy tissue demand for fuel, and thus demonstrates the adaptation which promotes optimisation of energy partitioning and growth efficiency (Jobling, 1997).

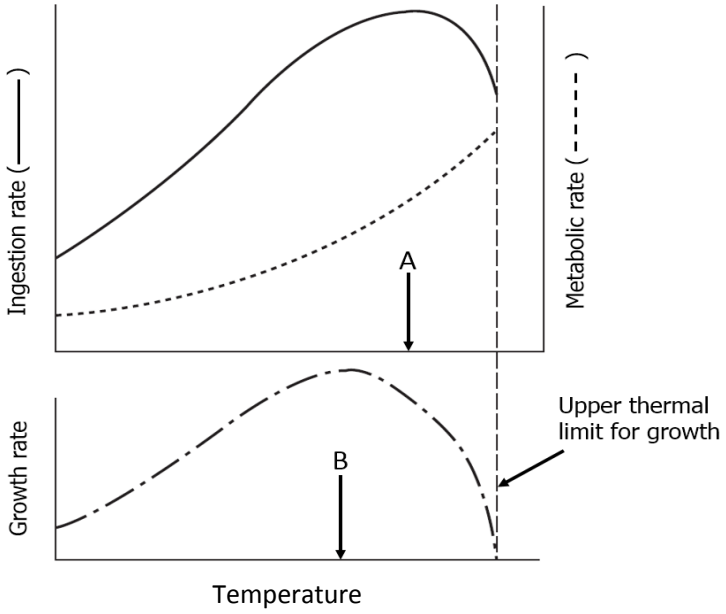

Figure 1.1 Rate – temperature curves depicting the effects of temperature on rates of ingestion, metabolism and growth. Temperature at which ingested rate reaches its maximum (A) is a few degrees higher than the optimum temperature for growth. Image sourced from Jobling (1997).

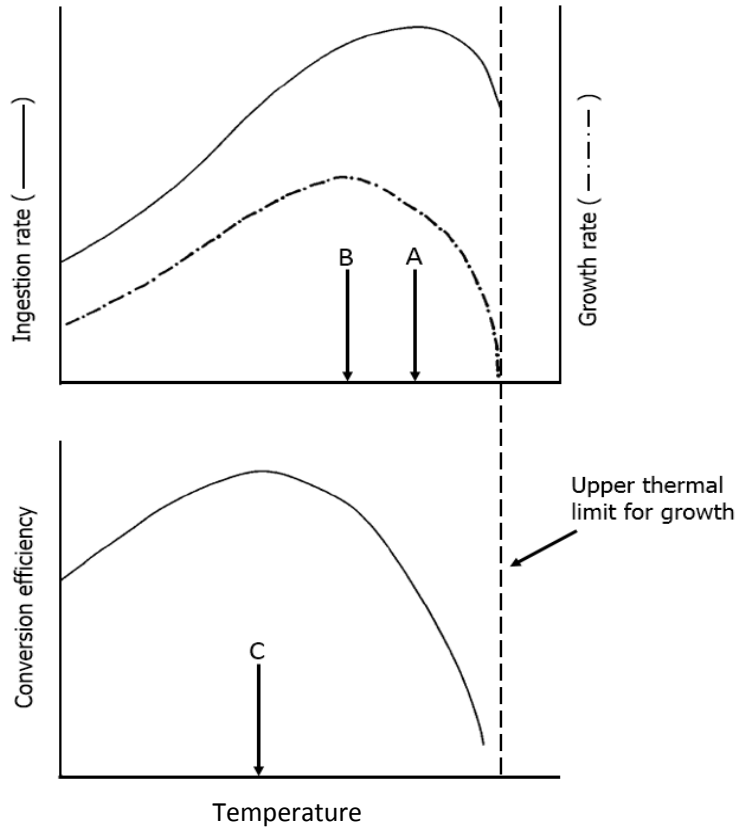

Figure 1.2 Rate – temperature curves depicting the effects of temperature on rates of ingestion and growth, and impact of temperature on conversion efficiency. Conversion is identified as growth per unit food ingested. Most efficient conversion (C) is achieved at a lower temperature than those at which ingestion (A) and growth (B) rates are at their respective maximum. Image sourced from Jobling (1997).

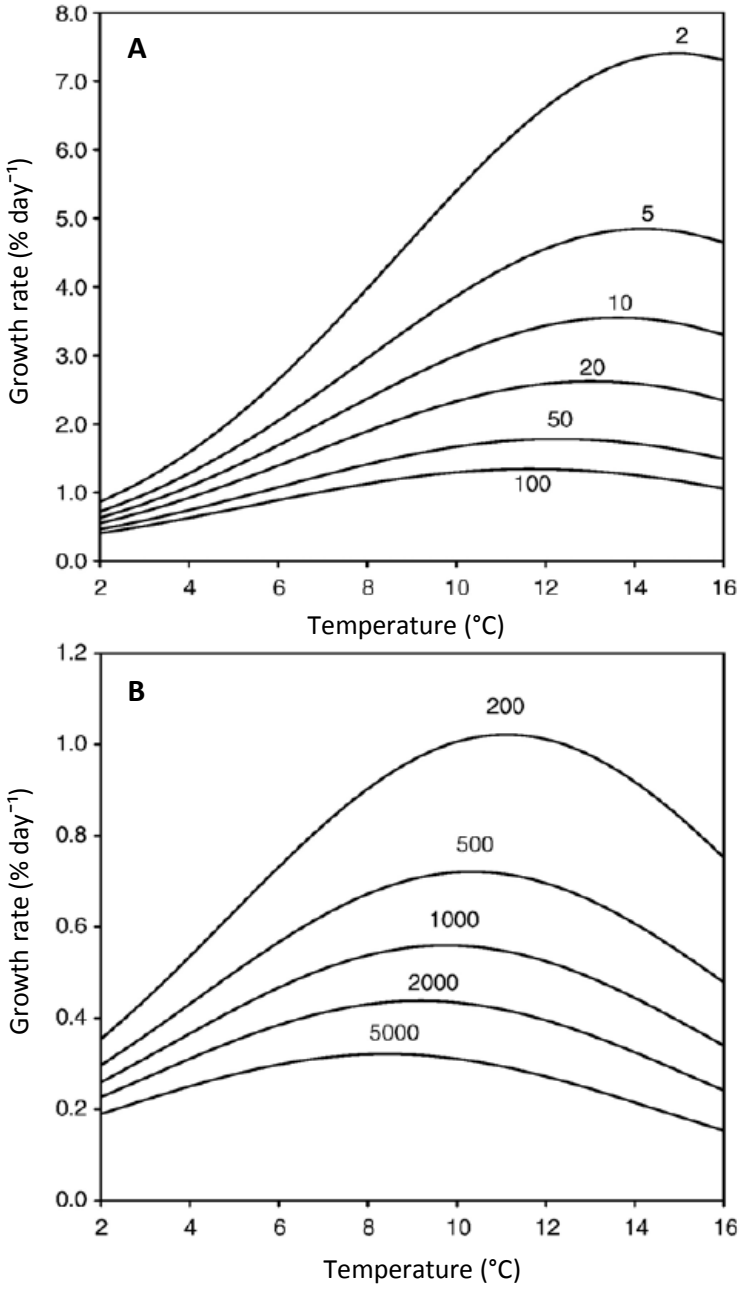

Figure 1.3 Model calculation of specific growth rate of Atlantic cod (*G. morhua*) for fish of mass 2, 5, 10, 20, 50 and 100 g (A) and 200, 500, 1000, 2000 and 5000g (B). The model depicts that when temperature increases the growth rate of juvenile cod first accelerates and then decelerates until the maximum rate is reached at optimal temperature. Note that the growth rates at each temperature decline with increased fish mass. Image sourced from Björnsson et al. (2007).

1.6 Test species

Two native New Zealand finfish species: snapper (Sparidae: *Chrysophrys auratus*, Fig. 1.4) and yellow-eyed mullet/YEM (Mugilidae: *Aldrichetta forsteri*, Fig. 1.5) have been chosen as test species for this thesis. The rationale behind the selection is at least threefold – firstly they both share (at least for some life stages) the same coastal-inshore habitats where they may experience similar environmental biotic (food availability, interspecific competition, predation pressure) and abiotic (seasonal temperature, light quality and quantity, oxygen and salinity oscillations) conditions; secondly despite sharing the same environment their life style/behaviour and therefore possibly their specific physiology traits are considerably different, which make them ambassadors of two relatively contrasting finfish groups; and thirdly a commercial (Fig. 1.6) and recreational interest for snapper is well established in New Zealand (Foscarini, 1988; Hartill et al., 2007; Mosmman, 2008; Parsons et al., 2009; Parsons et al., 2011; 2014). The size of the commercial snapper fishery is currently just over 6,000 tonnes per annum, while for recreational and customary fisheries less than 4,000 tonnes estimates have been reported (Ministry for Primary Industries, 2017). An accumulated knowledge makes a solid base for sustainable exploitation of snapper stocks, although the strategies for additionally required production enhancements need much better understanding (Parsons et al., 2014). On the other hand, YEM are listed as one of New Zealand fisheries (e.g. Ministry for Primary Industries, 2014. Fig. 1.7) and are often recreationally targeted (Taylor and Paul, 1998; Sullivan et al., 2005), but despite having exceptional growth potential (Coubrough et al., 2004), its full exploitable capacity has not yet been much investigated. The scale of YEM fishery (~20–30 and 5–10 tonnes per annum for commercial and recreational catch estimates respectively, Ministry for Primary Industries, 2014) is markedly smaller reflecting its current minute importance for New Zealand economy.

1.6.1 Snapper – general biology and taxonomy

Snapper (Fig. 1.4), sometimes known as red bream, is a member of the family Sparidae that is constituted of 33 genera and 110 species with a widespread distribution throughout tropical and temperate waters of the world oceans (Carpenter and Johnson, 2002). Snapper are commonly found in the subtropical and temperate Western Pacific where they are appreciated as an economically and recreationally important catch (Paulin, 1990). In New Zealand, snapper are an abundant species in north and north-east regions (Francis, 1994a; Willis et al., 2003) and they are a highly sought-after species with a long history of exploitation dating from the first Maori settlers 700 year ago (Parsons et al., 2014). In terms of habitat preferences, they are a demersal finfish that dwell mostly in soft

bottom and rocky reef habitats (Willis et al., 2003). Their distribution extends over the continental shelf, inhabiting shallow estuarine environments as juveniles until an ontogenetic habitat shift, at length of approximately 70 mm (Parsons et al., 2013) takes place and they move into deeper coastal waters ranging from 50 to 200 m (Francis, 1993). Even though they are not strictly territorial fish, they exhibit site fidelity behaviour (Parsons, 2003; Jackson et al., 2010). Ontogenetically, all snapper after hatching start as potential females (i.e. protogynous hermaphrodites) and once they reach approximately 20–30 cm fork length (Francis and Pankhurst, 1988) mature into adult males and females, which corresponds with 3–5 years of age (Francis, 1994a, Jackson et al., 2010). Snapper possess acute vision (Robinson et al., 2011) that assists their diurnal life style as an opportunistic predator (Hartill et al., 2003). In addition, they are considered to be shoaling long-lived finfish that can reach over 60 years of age and grow in excess of 100 cm in length (Parsons et al., 2014).

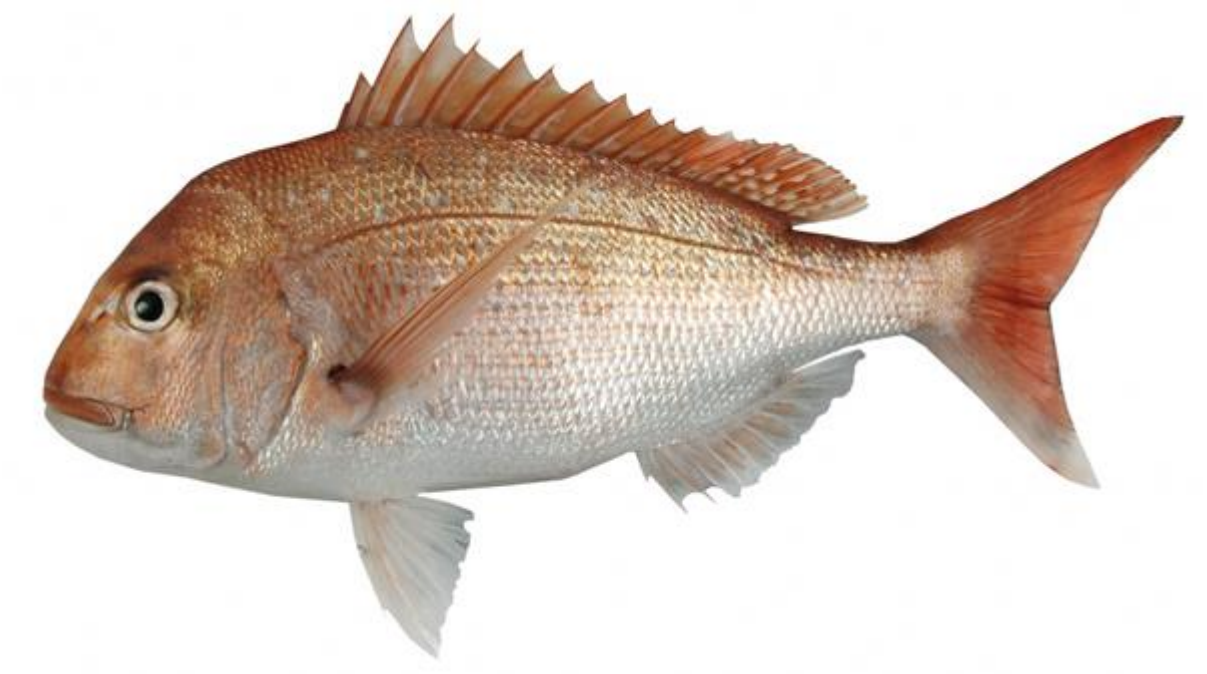

Figure 1.4 Adult snapper (*Chrysophrys auratus*), image sourced from:
https://www.mpi.govt.nz/travel-and-recreation/fishing/fish-species/snapper/

1.6.2 Specifics of snapper growth

Snapper have been a focus of scientific research in regards to commercial exploitation for over a century (Foscarini, 1988). One of the main aims was to explore growth parameters in both natural and captive environments (Bell et al., 1991). In Australasia snapper were considered as a solid candidate for aquaculture since they readily reproduce in captivity and are a highly desirable retail commodity (Smith, 1986). However, when it was first considered for culture, a potential challenge to the

production was posed by the apparent slow wild growth rates (Crossland, 1977). This has been disputed by Bell et al. (1991) whose findings indicated that, as was previously shown in Japan for the sister species *Pagrus major* (Foscarini, 1988), cultured snapper can reach a weight of up to 500 g (equivalent to 250 mm fork length – the distance from the tip of the snout to the middle posterior fork point of the caudal fin) within 12 months compared with wild fish that achieve 250 mm in length between 3 and 5 years (Crossland, 1977; Bell et al., 1991). In addition, according to Bell et al. (1991) growth can be even further increased with implementation of advanced technologies.

According to numerous studies (Francis, 1994a; Fielder et al., 2002; Cook et al., 2003) growth of wild snapper is explicitly seasonal. In other words, growth has a strong seasonal correlation with annual fluctuations in sea water temperature, where the summer period is linked to intense growth while growth rates during winter are markedly lower or growth may even stop or become negative (Francis, 1994a; Cook et al., 2003). Growth rates in wild snapper may also vary between regions and even amongst individuals of the same populations (Paul and Tarring, 1980; Francis, 1994a; Jackson et al., 2010). For an accurate estimation of wild snapper growth rates, which is important for comparability purposes with cultured stock, integration of several methods is recommended (Francis, 1994a). Francis (1994a) integrated two approaches: length-at-age measurement to estimate growth rates of cultured juvenile snapper, and a combination of length-frequency data and length-at-age data determined by counting daily increments on ear bones (otoliths) were used to determine estimates of growth rates of wild juvenile snapper. Furthermore, Francis (1994a) showed and Sim-Smith et al. (2013) recently supported that after metamorphosis, both wild and cultured juveniles grow linearly at an average rate of 0.7 mm day^{-1} for up to the first 100 days in northern New Zealand waters. After that a strong boost in growth is evident (Foscarini, 1988), and it can be attributed to an increase in thyroxine secretion (Kimura et al., 1992). Growth starts slowing down after 6 months of age, and gradually slows further until snapper reach ~ 125 mm fork length at 1 year of age, 180 mm on average after 2 years, 230 mm after 4 years and continue to decelerate further as they age (McKenzie et al., 1992; Walsh et al., 2006).

1.6.3 YEM – general biology and taxonomy

YEM (Fig. 1.5) or colloquially, but erroneously, known as herring or sprat belong to the family Mugilidae. They are the only member of the genus *Aldrichetta* and share New Zealand waters with another mugillid – cosmopolitan flathead grey mullet (*Mugil cephalus*) (Taylor and Paul, 1998). Distribution-wise, YEM can only be found around New Zealand (from Stewart Island to North Cape), southern parts of eastern and western Australia, southern Australia and Tasmania, and Norfolk Island where they are commonly seen in all types of costal habitats especially in estuaries, harbours and

lowland rivers (Curtis and Shima, 2005). They spawn in coastal water up to 30 km offshore so their early life stage (presettlement juveniles) is pelagic with regular schooling formation and exhibition of neustonic (i.e. living in the water surface) lifestyle (Coubrough et al, 2004). As juveniles they move towards more sheltered areas in estuarine and inshore environments (Coubrough et al, 2004). In terms of feeding YEM are omnivorous consuming both plankton and detritus in the water column and also algae, crustaceans, molluscs and similar items from the seabed (Curtis and Shima, 2005). They have a relatively short lifespan, which normally does not exceed 7 years; therefore, their sexual maturation occurs earlier than in snapper, which is at around 2 years of age or when they arrive at around 220 mm fork length (Taylor and Paul, 1998). Adults may often easily be separated between different ages as younger fish prefer more brackish and shallower waters. The ecological importance of this species in relation to its habitats seems to be very high since the entire ecosystem meets with YEM in the middle, i.e. in the top-down control YEM are a major food resource for predatory fish, sea-birds and sea-mammals, and in the bottom-up through their omnivorous feeding style they keep algae and small invertebrates in ecological balance and therefore they may be considered as an estuarine key-stone species (personal observation); however, such a comprehensive ecological investigation has not yet been conducted.

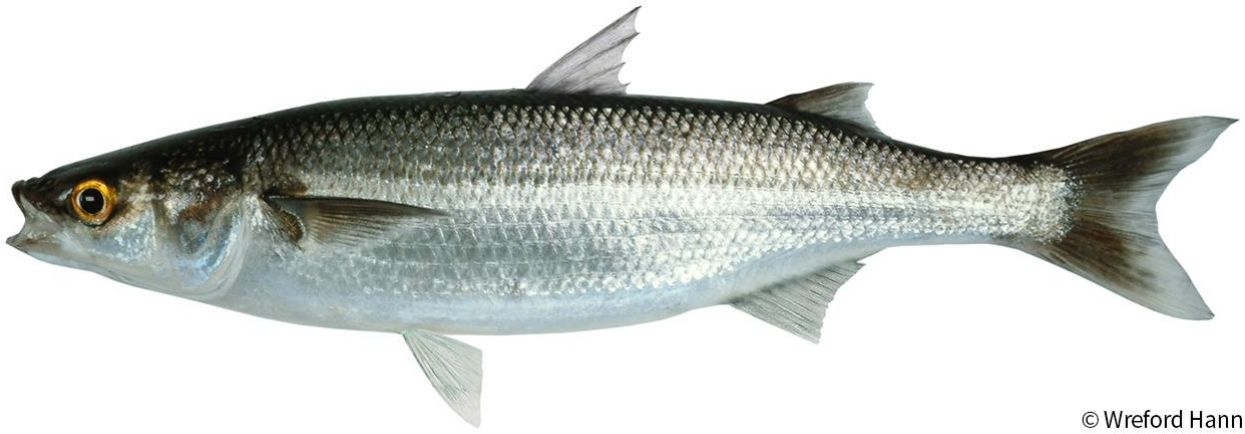

Figure 1.5 Adult yellow-eyed mullet (YEM, *Aldrichetta forsteri*), image sourced from: http://bestfishguide.org.nz/seafood/yellow-eyed-mullet/

1.6.4 Specifics of YEM growth

Information on growth parameters of YEM is somewhat limited. However, a good overview of geographical and sex-specific growth of YEM from New Zealand waters is presented in Curtis and Shima (2005). Their main findings can be summarised as heterogeneous growth rates of YEM amongst different locations and sexes. The method they utilised to determine growth rates was similar to Francis (1994a) – by extracting otoliths of wild mullet and validating their daily and annual increments. The values were used to construct size-at age curves from which further estimates of growth rates were made. Their results suggested that males are overall slower growers than females at the lowest sampling latitude in both the North and South Islands; however, this pattern seems to progressively reverse with higher latitudes. Furthermore, growth in their study, due to similarity in distributions among all sampling sites, was best explained by the linear model. In the past, several authors stated that the maximum lifespan of YEM can be observed at 6 (Bradstock, 1985) or 7 years (Lenanton and Potter, 1987; Potter et al., 1990). The largest mullet Curtis and Shima (2005) sampled at each site were correlated with the proposed oldest ages for YEM. This observation supported the opinion that YEM may belong to a group of minority fish species that exhibit non-asymptotic growth and it was suggested that YEM possibly choose to maximize their lifetime fitness by continuously devoting high levels of available energy into somatic growth (Potter et al., 1990; Curtis and Shima 2005).

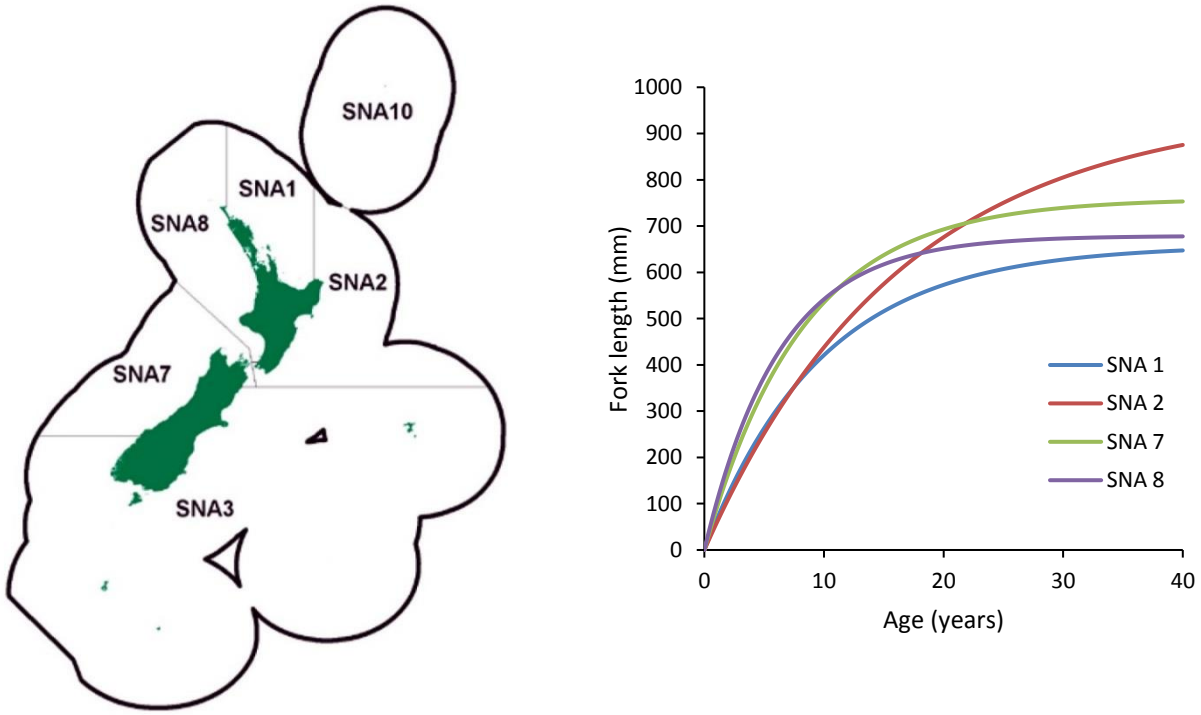

Figure 1.6 Six snapper fishery management areas according to MPI (Ministry for Primary Industries New Zealand, left diagram), with von Bertalanffy growth curves fitted to parameters reported in the SNA May 2016 Fisheries Plenary Report (Ministry for Primary Industries, 2016; right figure).

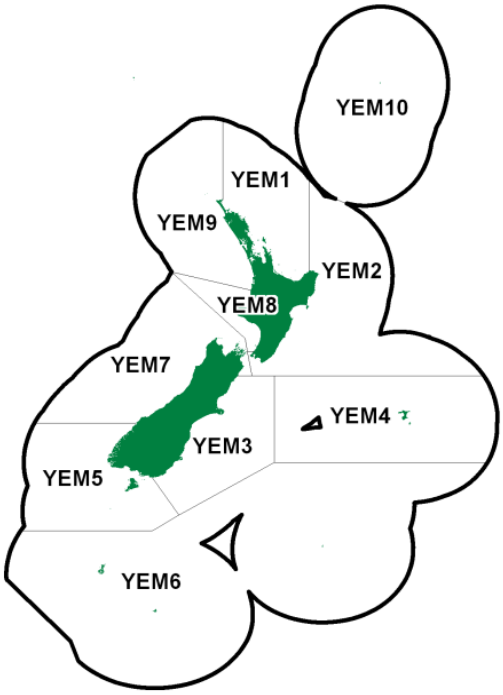

Figure 1.7 Ten YEM fishery management areas according to MPI (Ministry for Primary Industries New Zealand).

1.7 Objectives of the thesis

The aim of this thesis was to acquire information that can fit into the big picture of understanding fish growth physiology so it is:

- available to other disciplines as a foundation for further investigation into enhancement of production in the commercial sector

- added as new knowledge to the global data base and to use for academic purposes

- available for conservation purposes, particularly in relation to global change, provided that extrapolations could be made to other phylogenetically related species.

More specifically, this aim was addressed by employing two native New Zealand finfish species that are characterised by different lifestyles, and by using them the intention was to investigate physiological traits that support their coexistence, development and growth. Moreover, this thesis investigated what could be their maximum growth performance under their current genetic makeup

and how they do it from energy partitioning and a biochemical point of view. By implementing an *ad libitum* feeding regime it was anticipated to observe and address both the what and how questions. This is because under the unlimited food supply, food, as a likely growth limiting factor for the test species in the wild, was excluded from influencing growth traits in a way that would be expected in the natural environment and from that perspective growth could be maximised.

This in turn should have maintained physiological pathways associated with growth running at highest levels throughout the entire experimental period of 12 months, but still shaped by environmental conditions (e.g. temperature and light). Therefore, it was expected to observe trajectories of these pathways in a relatively clear fashion. It is believed that this permitted more precise conclusions about test species growth potential and related physiology and biochemistry. At the same time the effects of both food limitation and temperature as leading growth driver would be easier detected and more accurately explained. The final question of interest was to see what difference a food unlimited diet can make, if food is indeed limiting in natural habitats, by comparing experimental findings with data from wild populations.

In compliance with the above description four main areas of investigation were designed to address the outlined requirements of this thesis. They were: 1) food unlimited tank array, maximum potential growth rate experiments, 2) biochemical profiles of selected tissues from specimens originated from tank array experiments, 3) separate sets of experiments based on respirometry procedures to investigate metabolic phenotype of the two test species and subsequently indirect proxy for growth termed specific dynamic action (SDA, energy expenditure due to ingested meal) and 4) resourcing wild fish growth data and comparison with tank array findings.

These four areas were split into six chapters to follow a logical explanatory course, and the objectives are summarised here:

- Chapter 2 objectives: this chapter dealt with the tank array experiment and supplementary organ morphometrics (i.e. body indices). The tank array growth experiment was the carrier of the main thesis theme, and the focal aim was to characterise the growth profile of the test species by determining their maximum potential growth rates under *ad libitum* diet conditions conducted for the period of one calendar year. Concurrently, this chapter sought to monitor fish condition and mass – length relationship throughout the course of the experiment.

- Chapter 3 objectives: this chapter was based on respirometry work, which dealt with aerobic metabolic activity of the test species. Resting metabolic rates (the closest approximation of basal or maintenance metabolic rate) and maximum metabolic rates (highest rate at which oxygen can be consumed) were determined in order to produce aerobic scope (a capacity of an organism to perform any aerobic functions such as growth, digestion, reproduction and locomotion) and to see to what

extent the scope was shaped by temperature that mimicked the temperature range experienced by fish in the tank array trials. These rates were also later utilised as a framework to fit data from chapter 4.

- Chapter 4 objectives: in this chapter the main focus was to deal with fish metabolic response to an ingested meal (SDA). Therefore, the aim was to determine the properties of SDA of the test species as well as to evaluate how much aerobic scope or aerobic capacity was occupied during digestion of a meal, or in other words how much aerobic capacity was still available for other activities.

- Chapter 5 objectives: this chapter sought to assess the biochemical profile of the test species by determining annual activity dynamics of enzymes known to correlate with growth parameters and/or are important catalysts in main biochemical pathways (i.e. cytochrome c oxidase, citrate synthase, pyruvate kinase, lactate dehydrogenase, 3-hydroxyacyl-CoA dehydrogenase, trypsin and chymotrypsin). Another aim was, concurrently with enzyme activities, to determine concentrations of key tissue metabolites, lactate and glucose, and the energy store compound glycogen, which further aided in evaluation of metabolic pathways involved during the intense growth of test fish through the period of 12 months on unrestricted diet.

- Chapter 6 objectives: Overall growth progress after 12 months of the food unlimited experiment was examined for any differences between cultured growth rates achieved during feeding trials and growth rates of wild fish stocks, where the case of higher growth in the cultured fish would have pointed out to the fact that food may have been limiting in the wild. Therefore, the objective of this chapter sought to determine these differences, first by finding relevant data for the comparison which were obtained from two sources – from surveyed wild fish and from published literature, and then compared them against each other. Finally, this chapter was aimed to examine how two main environmental variables, namely sea-water temperature and food availability (represented with chlorophyll *a* concentration as a proxy for net primary production) affected wild snapper growth rates.

- Chapter 7 objectives: In this chapter growth strategies from the test species were compared and key differences discussed. In addition, aspects of fish physiology portrayed in chapter 3 and 4 were further discussed in the light of capacity for growth. And finally, some implications of the present work were proposed and ideas for further research development of the elaborated topics were projected.

CHAPTER 2

Annual growth profile – seasonal effects on growth rates, condition and supplementary morphometrics of two coastal temperate species

2.1 Introduction

2.1.1 The temperate environment – how does it shape fish feeding behaviour and growth performance?

Temperate environments are where seasonal variation in climate variables are predictable and where frequency and duration of typical seasons are enveloped within annual cycles (Shuter and Post, 1990; Gotthard, 2001). Since pronounced variations in biotic and abiotic environmental variables typify the temperate zone, organisms and particularly ectotherms have adapted to those cyclical changes and developed strategies to maximise their fitness and survival (Gotthard, 2001). The environmental variables that exhibit the most prominent seasonal change in these habitats are temperature, light and food availability (Pickett and Pawson, 1994; Sánchez-Vázquez et al., 1998; Jobling, 2008).

2.1.1.1 Seasonal effects on food intake and growth in temperate environments

In order to feed, fish rely on sensory abilities to recognise, capture and ingest food items, and a physiological capacity to digest and utilise the ingested nutrients, which all depend on environmental factors that could influence a single or multiple stages in the feeding process (Kestemont and Baras, 2001). Of these environmental factors, temperature is known to affect food supply, probability of food capture, digestion physiology, absorption and nutrient conversion, all of which consequently affect growth (Kestemont and Baras, 2001). Colder winter seasons are associated with a shorter daily photoperiod, which also potentially mediates depressed food intake and therefore growth. Annual increases in ambient temperature that coincide with the lengthening of the photoperiod, from spring towards summer, is characterised by increased feeding activity, elevated feed intake and growth rates

which are generally retained over the warm summer (Higgins and Talbot, 1985; Smith et al., 1993; Tveiten et al., 1996). Therefore, it seems that temperate fish employ seasonally changing environmental factors to time their feeding behaviour, growth and reproduction within predictable annual cycles (Houlihan et al., 2001). In addition, any abrupt changes to those factors, especially temperature, irrespective of an increase or decrease may cause drastic feed intake reduction (Alanärä, 1992b). This emphasises temperature and photoperiod as major factors governing fish feeding behaviour and growth performance providing that food supply is abundant/adequate (Karaås, 1990; Griffiths and Kirkwood 1994; Jobling, 2008).

Food abundance is an additional major growth driver in temperate environments since it could be spatially unpredictable or patchy (i.e. oscillating between abundance and scarcity) and temporally variable (i.e. seasonal availability of certain prey types) and so influence seasonal changes in phasing in the feeding rhythms (Houlihan et al., 2001; Madrid et al., 2001). Furthermore, seasonal changes in environmental cues as well as prey type is often synchronised with pulses in planktonic production (Edwards and Richardson, 2004), which also indirectly governs fish migratory behaviour as an important activity for matching fish spawning time with seasonal peak in productivity to also support rapid growth of fish progeny (Sims et al., 2004). In temperate reef environments, where growth of juvenile fish is mostly limited by food availability (Jones, 1987; Sogard 1992) or hierarchical social inhibition (Tupper and Boutilier, 1997), rapid growth during the summer was found to be especially advantageous since larger specimens normally have greater overwintering survival rates (Henderson et al., 1988). In contrast, fish in fresh waters do not always follow the feeding behaviour pattern frequently observed in oceans since in riverine temperate environments food availability is often related to a change in seasonal timing in flow or flooding regime rather than on increase in water temperature and photoperiod (Bacon and Heagney, 2001; Tonkin et al., 2011).

2.1.1.2 Seasonal effects on fish condition and energy partitioning

Fish condition and specifically energy stores *per se* were proposed to be important internal cues for regulation of feeding behaviour and they were particularly linked with the reduction in feed intake when energy reserves were accumulated to optimum levels (Tveiten *et al.* 1996). Following replenishment of stored resources, feeding intensity is expected to decrease causing growth rates to slow down (Broekhuizen et al., 1994; Jobling and Johansen, 1999). This change in behaviour may be regulated by specific metabolic signals produced by different tissues via the central nervous system facilitating constant assessment of the status of the energy reserves (Kiess et al., 1999; Magni et al., 2000).

Seasonal variation in condition is often reported for many temperate fish species, which in turn is highly correlated with feed intake and amounts of accumulated energy stores (Blackwell et al., 2000). For juvenile fish it is generally thought that optimising survival is first characterised by striving to out-grow predation risks by maximising growth in length. However, at the right time the energy allocation should be switched towards energy reserves accumulation to endure challenging overwintering conditions (Post and Parkinson, 2001; Diaz et al., 2009; Sim-Smith et al., 2013). During summer, juvenile fish use all available energy and direct it into maximising growth (Hurst and Conover, 2003; Biro et al., 2005; Huss et al., 2008), while during autumn more of that energy goes towards accumulation of energy reserves (Hurst and Conover, 2003; Biro et al., 2005; Huss et al., 2008). In addition, it has been noted that throughout the year protein accretion may be more or less positive, while quantities of body lipids often show extensive seasonal variations (Jobling, 2008). This change in body composition for temperate or high-latitude fish is directed towards accumulating energy reserves when conditions are favourable in the year that can be subsequently mobilised as a metabolic fuel when external energy input during winter is low (Elliott, 1994; Jobling, 1994). Behavioural and physiological responses of temperate fish during the warmer months of the year bear a resemblance to fish that were under experimental conditions fed *ad libitum* after a period of starvation where they had pronounced hyperphagia (increased food intake), growth and condition enhancement, and the rapid replenishment of energy reserves (Jobling and Johansen, 1999; Sæther and Jobling, 1999).

2.1.2 Seasonal effects on organosomatic indices and additional morphometrics

Organosomatic indices, commonly expressed as percentage mass of visceral organs or tissues relative to total or gutted body mass (i.e. ratios), are quickly obtainable indicators of change in the nutritional and energy status of fish (Goede, 1990). Organosomatic indices often follow, with a shorter or longer time lag response, change in environmental conditions (Goede, 1990). The most common organosomatic indices of interest in juvenile fish growth and development research are hepato-somatic (HSI), spleno-somatic (SSI), cardio-somatic (CSI), entero-somatic (ESI), visceral lipid (i.e. mesenteric fat deposits, VLI), and relative gutted mass or gutted mass index (GMI).

HSI is often found to increase due to deposition of energy reserves when food becomes more available. In temperate fish it may have a seasonal character, or it may increase when the ration has been enlarged and therefore for both cases it has been reported that it could closely correlate with growth rates (Carter et al., 1998; Goa et al., 2012; Hauser-Davis et al., 2012). This is because liver is considered to be a major organ to store energy matter for many fish either in a form of lipids (e.g. Atlantic cod, *Gadus morhua*; Dutil et al., 1995) or glycogen (e.g. snapper, *Chrysophrys auratus*; Booth et al., 2006).

The spleen is unique to vertebrates, functioning as an important blood store and filtering hub that is also responsible for adaptive or specific immune responses, hence is regarded as a major lymphoid organ in fish (Mebius and Kraal, 2005; Kurz et al., 2007). In addition, spleen mass was found to be linked to fish health status (Haididi at al., 2008; Wiens at al., 2013). Furthermore, Wiens at al. (2013) reported heritability traits connecting spleen mass with specific disease resistance in a teleost fish, therefore SSI can be considered as an indicator of fish health and wellbeing.

CSI as an indicator of cardiac development (Helland et al., 2009) may alter with sexual maturation, since it has been shown that mature male rainbow trout (*Oncorhynchus mykiss*) had a significantly larger CSI than immature males or females (Franklin and Davie, 1992); or with changes in environmental conditions (Aho and Vornanen, 2001). In particular, acclimation to colder temperatures in many teleosts is associated with an increase in relative heart mass, which is partially due to an increase in blood viscosity that requires enhanced contractile heart action (Aho and Vornanen, 2001). Therefore, annual CSI changeability may have a seasonal character.

ESI is mainly determined in relation to activity of mitochondrial enzymes as indicators of growth rates (Pelletier et al., 1994; Guderley et al., 1996). Generally, it is believed that the length of intestines is determined genetically since it is not common to observe major differences in intestinal dimensions (length) between conspecifics of similar size and age (Weatherley and Gill, 1983; Pelletier et al., 1994). This implies that when more food is available the increased demand to process an ingested meal may be met by enhanced enzymatic activity rather than by an increase in gut size as a mechanism responsible for improved absorption and ultimately growth rates (Pelletier et al., 1994). Despite this notion, prolonged periods of plentiful food supply, due to faster increase in body mass relative to intestinal mass growth, may cause ESI to drop (Guderley et al., 1996; this study), therefore a seasonal oscillation in ESI may be observed.

VLI, a metric with often pronounced seasonal dynamics, is an indicator of energy resources and accrued lipid stores (Nelson and Magnuson, 1992; Mackereth et al., 1999). This can be translated into the fish's capability to survive during periods of food deprivation as well as investment into reproductive fitness (Nelson and Magnuson, 1992; Niva, 1999). VLI is therefore regularly correlated with fish condition and even more, condition is frequently better explained with VLI than with muscle growth, making it often a prevailing predictor of growth status and condition (Gauthier et al., 2008). Nevertheless, visceral lipid deposits can fluctuate depending not only on seasonal effects but also on other variables such as sex, fish size and stress level (Adams et al., 1993).

When it comes to commercial investigation of fish stocks, a gutted mass is a better representative of fish growth as an indicator of muscle mass increase than full wet mass, since the viscera are not of

the same economical value (Lanari et al., 1999). Therefore, gutted mass is often a morphometric associated with growth studies in applied physiology or fishery sciences and it may be accompanied with gutted mass index (GMI). GMI is especially informative for studies where fish are scrutinised either under full rations (*ad libitum* regime), fasting regime or a combination of the two. This is because mobilisation of energy stores and metabolic activity in muscle proteins can be observed through change in GMI as it follows the condition index with a short time lag, indicating the period required for the ingested food to be converted into muscle mass. Hence, GMI may also show seasonality in its annual trajectory (Rueda et al., 1998; Everson, 2008).

2.1.3 Test species – juvenile snapper and YEM in seasonal/temperate environments

2.1.3.1 Snapper

The natural bio-geographical range of snapper (*C. auratus*) extends from tropical/subtropical areas of eastern and western Australia and western Pacific Islands (~ 15 °S) to temperate areas of Tasmania and the North Island and northern part of the South Island of New Zealand (~41 °S), with occasional occurrences in southern South Island down to 45 °S (Crossland, 1981; Paulin, 1990; Gomon, 2008). Since snapper used in this study originated from Tasman/Golden Bay area located at the northern tip of the South Island, they can be regarded as a population adapted to the southern boundary of the species range. They experience greater oscillations in photoperiod and overall lower temperatures [i.e. minimum daily average in winter ~10°C and maximum of ~22°C in summer (data from Plant & Food Research monitoring station, Nelson)] compared with ~20 to 28°C experienced by conspecifics from the northern boundary of their natural distribution (Crossland, 1981; Willmer et al., 2004). Juvenile snapper are considered to fall within a range of 10–230 mm fork length at ages between 0+ and 3+/4+ (Parsons et al., 2014). Small juveniles are commonly present in shallower waters of estuaries, harbours and bays between late spring and early autumn in association with a structured habitat type that provides refuge and shelter, higher abundance of food, potentially warmer and often less saline environment – conditions associated with rapid growth performance (Usmar, 2009; Sim-Smith et al., 2012; Lowe, 2013). Before winter, at about 70 mm fork length they start moving to deeper coastal environments, but both older juveniles and adults are known to return to shallow coastal habitats, presumably to utilise the prey rich warmer inshore waters (Francis, 1994a; Parsons, 2016). This temporal and spatial change in snapper abundance implies that apart from ontogenetic migrations, seasonal offshore–inshore movements also occur (Parsons et al., 2014). In keeping with this behavioural pattern, it is not uncommon to encounter more larger juveniles or young adult snapper in Nelson Haven (estuary of the Maitai River, north of South Island, New Zealand, where this

study was situated) during the warmer months but not during winter (personal observation and common local knowledge). Therefore, snapper at the study location can be considered as transient residents.

Generally, snapper have a typical annual/seasonal growth pattern, as observed for many temperate fish, where rapid growth characterises summer, while growth reduction characterises winter. Overall lifetime growth has an asymptotic trajectory (Francis, 1994a; Jackson et al., 2010).

2.1.3.2 YEM

Yellow-eyed mullet have a narrower and more southern latitudinal band (~28–48 °S,) as its natural distribution range (http://www.fishbase.de/summary/Aldrichetta-forsteri.html). YEM may experience temperature fluctuations from ~5 (winter) – 15°C (summer) at the southern edge to ~15–25°C at the northern periphery of their distribution which extends from subtropical to sub Antarctic regions (McDowall, 1978). They are considered euryhaline species (Taylor and Paul, 1998) and classified as temperate marine estuarine-opportunists (Chub et al., 1981; Curtis, 2003). YEM are permanent residents in shallower coastal/estuarine environments, which imposes necessary physiological adaptation to large daily and seasonal fluctuations in ambient temperatures. Furthermore, they are short-lived, relatively early sexually matured, which all require fast and steady annual and inter-annual growth, and thus are probably tolerant to temperature fluctuations (Chubb et al., 1981; Coubrough et al., 2004). YEM, as other members of the Mugilidae family, often show an inclination for low saline and fresh water environments as juveniles, which correlates with the fact that oligohaline (1.1–5.0 ppt) or mesohaline (5.1–15.0 ppt) waters support maximum growth performance of mugilids (Cardona, 2006). In addition, the abundant presence of juvenile YEM in riverine environments in spring and summer coincides with time of maximum algal growth, which is found to be the most common food item on the menu of YEM (Manikiam, 1963; Jellyman et al., 1997). Since YEM are considered to have mainly a temperate-water distribution, the greater occurrence of larger YEM in estuarine environments during cooler months, as opposed to summer suggests seasonal movement, implying that shallower coastal waters may become too warm for adult YEM (e.g. temperature in western Australian estuaries may average 27–30°C in summer) (Chub et al., 1981).

According to Curtis and Shima (2005) YEM inter-annual (lifetime) grow is atypical since it cannot be described as asymptotic, but is rather linear. In addition several reports (e.g. Chub et al., 1981) indicated the possibility of YEM continous annual growth even over the cooldest months suggesting a difference in the growth strategy of snapper and YEM.

2.1.4 Objectives of the chapter

Two temperate finfish species, snapper and YEM, have been suggested to use two different growth strategies which, when untangled, may help to better our understanding of ectothermic adaptation to temperate habitats. In addition, the knowledge of annual feeding and growth profile of selected species is also of particular interest as it can assist evaluation of their growth capacities and limitations as potential New Zealand aquaculture/stock enhancement candidates.

Recordings of growth variables (mass and length) and feed intake were carried out on the test species on a monthly or bimonthly basis under ambient environmental conditions with a feeding to satiation diet regime (an approach that controls for effects of potential food absence/patchiness in the wild). Therefore, the objectives of the chapter encompass characterisation of annual growth performance with emphasis on seasonal effects on growth and fish condition dynamics with a relation to seasonal feed intake responses and energy resource partitioning. Concurrent with monthly growth variables, information about organosomatic indices were also obtained to aid describing the condition and health status of the two species during the 12-month study.

2.2 Material and Methods

2.2.1 Husbandry procedures and animals used in the study

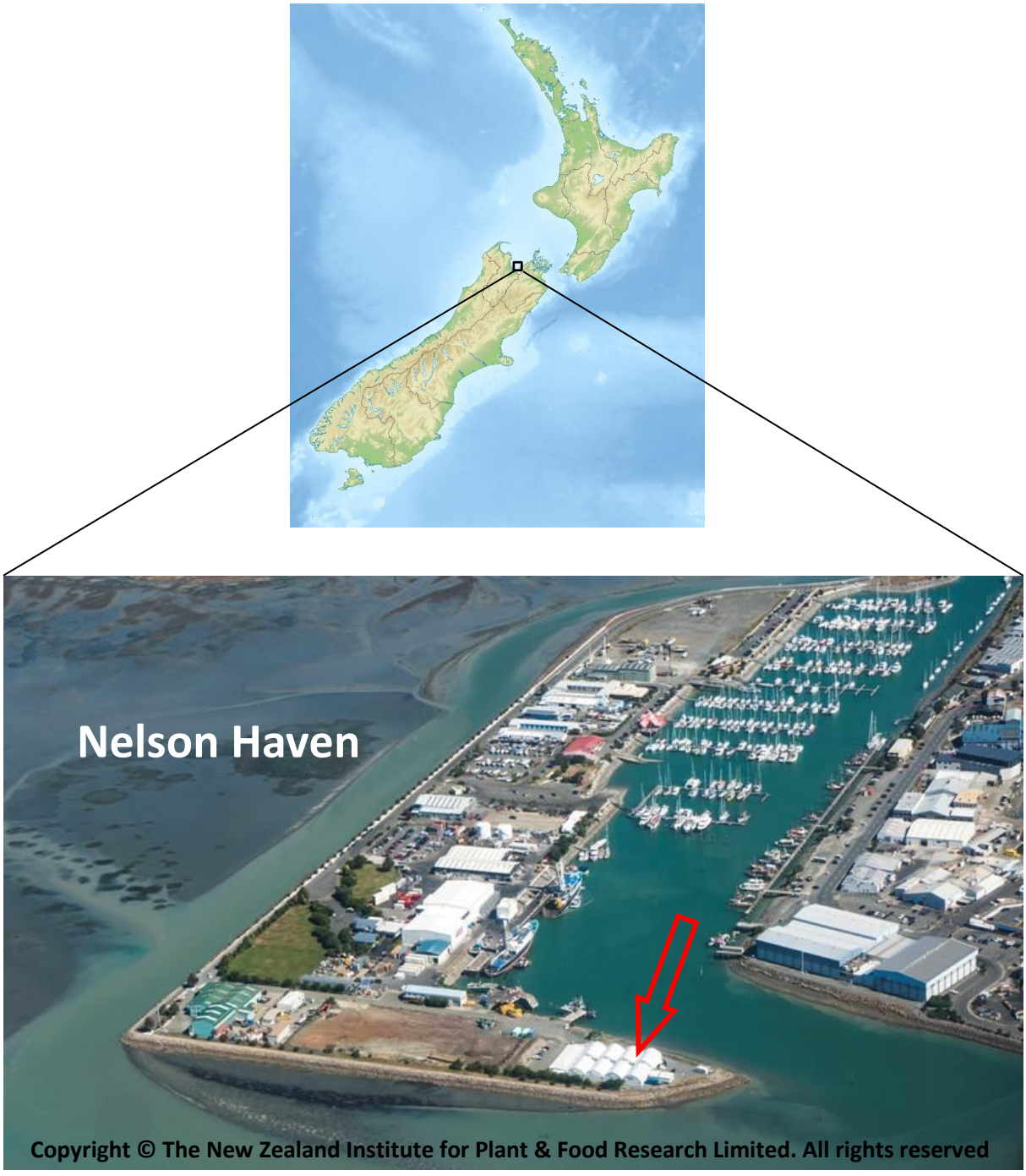

Figure 2.1. Location of PFR Nelson facilities in Nelson Haven (red arrow) where majority of experimental work was conducted from spring 2014 to summer 2017.

2.2.1.1 Husbandry procedures and tank set up

The unlimited food growth experiments were conducted at the Plant & Food Research facilities, Seafood Production Unit, Maitai site, 293 Akersten Street, Nelson 7010 (PFR) (Fig. 2.1). During the course of the experiments spanning October 2014 to November 2015 and March 2015 to April 2016 for snapper and YEM respectively, fish were kept in flow-through tanks (Fig. 2.2) The tanks used were 6000 L water tanks with 5000 L of operational capacity, with dimensions: 2510 mm in diameter and wall height of 1205 mm. Tanks were manufactured by RX Plastics LTD, Ashburton, New Zealand. The flow capacity of the flow-through system was maintained at 35–50 L minute^{-1} with water being supplied directly from the Nelson Haven, Tasman Bay. The Nelson Haven is the estuary of the Maitai River encircled by a 13 km Boulder Bank located at the North of the South Island (Fig. 2.1). Before entering the premises the incoming water was first accumulated in an artificially built aquifer situated in the vicinity of the PFR facilities (coordinates: latitude: -41.254412, longitude: 173.280118) and then passed through a natural sand filtering system. The filtering system ensures high water quality with stable water chemistry including salinity (i.e. ranging from 34 to 35 ppt) even during the strong elements of nature (i.e. storms and earthquakes). Storm events and spring low tides do increase turbidity of the water. However, changes in turbidity were not greater than what the fish would experience naturally, as based on comparison with the external feed station monitoring site. The pH of the incoming water may oscillate between 7.7 and 8.2 but, by means of air bubbling (via the aerating system) when entering the tanks, it is rapidly buffered to ~ 8.2 and maintained stable. Seawater enters tanks as a jet that propels circular motion of the water forming a constant current against which the fish swim. This keeps aggressive behaviour amongst fish and the occurrence of other negative spontaneous behaviour. Another purpose of the jets is to additionally aerate the water in the tanks. The main aerating system is based on a portable custom-made ring aerating system that ensured no less than 90% oxygen saturation in tanks at all times. An important aspect of the system is that the ambient temperature in the experimental tanks perfectly reflect seasonal changes in the Nelson Haven Estuary. This is important since one of the leading questions in this study is related to the seasonal temperature effects on growth of the test species. In terms of light conditions, the tanks were positioned in a bay where penetration of natural light was allowed through a white Duraflex cover (Redpath Pacific Ltd, Palmerston North, New Zealand). Duraflex allows 70% shade effect and preserves near full spectral profile in the bays mimicking outdoor light conditions. Tanks were cleaned twice a week and regularly monitored for signs of injured, dead or "jumper" fish (fish that jump out of the tank in incidences of increased turbidity or other reasons). Several times during the winter period, especially in snapper tanks, due to increased occurrence of skin lesions, formalin (i.e. water solution of the formaldehyde gas) treatments at 200 ppm for 1 hour were applied. The purpose of the treatment was to reduce numbers of pathogens that cause or support (via secondary infection) the formation and or proliferation of skin lesions.

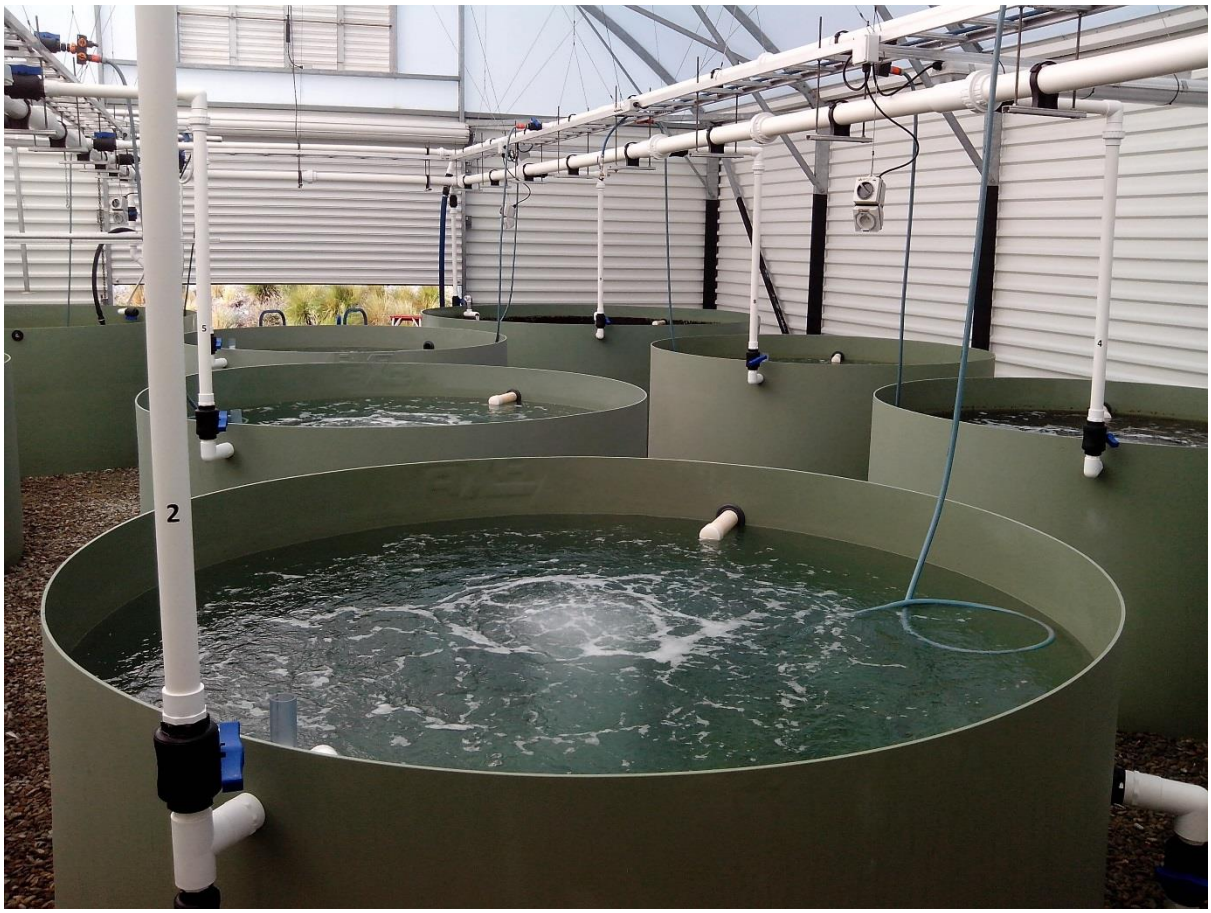

Figure 2.2. Flow-through tanks used for the unlimited food growth experiments at the Plant & Food Research facilities, Nelson.

For each of the test species two tanks (tank 1, T1 and tank 2, T2) were assigned for the experiment. They were located in a bay where there were 11 operational tanks in total. In the bay at least one tank was unused at all times. The unused tank/s served as the receiving tank/s. Whenever fish from any tank in the bay were scheduled for moving, either as a requirement of this study or as a part of the routine PFR operation, they were moved into one of the receiving tanks. The tank from where fish were moved then became the new receiving tank, and the one where fish moved into became their resident tank until the next scheduled sampling/moving day, and so on. Once fish were moved, the tank was drained, cleaned with a water blaster and left soaking in 250 ppm of 65% calcium hypochlorite overnight. The next day the chlorinated water was neutralised with 500 ppm sodium thiosulphate, drained and filled with fresh seawater and the new receiving tank was ready for the next fish to move in. The general practice was when fish were scheduled for a sampling/measurement session one tank was processed on the first day and the other tank on the second day. The tanks were

never processed and moved in the exact T1 first and T2 second order. From a practical point, the first tank moved was always the tank closest to the receiving tank. Concurrently, this practice also served to prevent any effects that may be imposed on the growth parameters by a consistent orderly fashion of moving tanks. Since the work requirements on the other resident fish (not related to the study) in the bay, were independent of the moving schedule designed for snapper and YEM in the growth experiment, it was always unknown which tank would become the receiving tank. Those unpredicted circumstances governed the randomised order of moving the experimental tanks per sampling/measurement session. Furthermore, in order to avoid the occurrence of the tank effect that may ensue if experimental tanks were located alongside the common-use walkways (Speare, 1995), the tanks utilised in the growth experiment were purposely chosen not to be associated with the main walkways.

2.2.1.2 Snapper

Snapper used in the growth experiment were hatched on 11th and 12th November 2013 at the PFR facilities in the marine hatchery unit. The eggs were produced by wild brood parents (i.e. captured and acclimated brood stock). For several months before entering the experiment, fish were kept on a maintenance diet composed of mixed pellets (2.3 and 3 mm Nova ME, Skretting, Australia) for four consecutive days and with in-house prepared alginate for three remaining days week^{-1} at a ration of 2% of estimated fish mass in a tank day^{-1}. They were fed three times day^{-1} by hand. At the age of ~11 months 352 snapper were selected for the experiment and split into two tanks. The experiment began when the first measurement session, which included tagging, took place on 8th and 14th October 2014 and it was terminated on 20th and 19th November 2015 for T1 and T2 respectively.

For average mass and length values with their associated standard deviations, stocking densities and numbers of tagged and untagged fish in T1 and T2 at the beginning and the end of the growth study refer to Table 2.1.

2.2.1.3 YEM

YEM for the food unlimited growth study were obtained from the wild by employing the in-house built round fish trap (Fig. 2.3). The trap (diameter 1.2 m, height = 0.8 m, volume = 900 L) has an 8-mm steel frame enclosed with 15 mm knotless mesh. There are two parts to the trap, which are secured with cable ties and at one end connected with hinges that allow the trap to open forming two half-

round compartments (Fig. 2.3B). Before deployment the trap was baited with 0.5 kg of fish mince and 0.5 kg 15 mm commercial fish pellet (Nova, Skretting Australia). The trap was deployed on 20th February 2015 in the Nelson Haven in the proximity of PFR facilities during low tide and immobilised by attaching to the soft sediment floor with sand anchors.

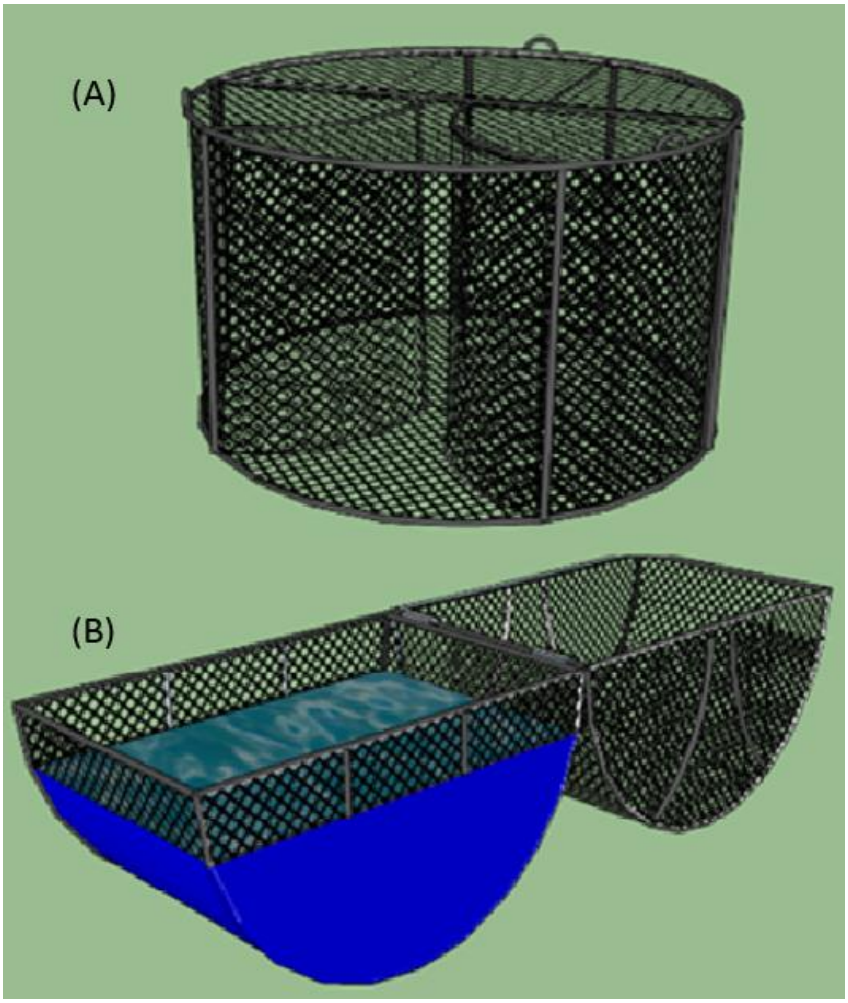

Figure 2.3. The round trap used to capture YEM. (A) Position of the trap during the deployment, (B) rotated and opened trap during the fish manipulation, blue indicates the shroud compartment. Image courtesy of Denham Cook, Plant & Food Research, Sea Food Research Unit, Nelson.

After ~6 hours during the ebbing tide the trap was retrieved and when still under the water one half was covered with a PVC shroud forming the shroud compartment. The trap was rotated so that the shrouded compartment was positioned in the water and the other compartment was above it. The trap was then opened allowing access to the shrouded part holding the captured fish in ~460 L sea-water. The sea-water in the shrouded compartment was separated from the sea and thus allowed for

administration of an anaesthetic (10 ppm AQUI-S, Appendix 1) required for sedation of fish before manipulation. A portable aeration system with a battery compressor and air-stone was utilised to provide substantial aeration of the compartment. Fish of 120–150 mm fork length (FL, the distance from the tip of the snout to the middle posterior fork point of the caudal fin) were selected as suitable for the study assuming they belonged to the first-year class according to Curtis and Shima (2005). Selected fish were carried by bucket to the transporting vehicle and placed into 70 L plastic crates (dimensions: 58 x 38 x 32 cm) filled with seawater supplied with additional aeration. On arrival at the PFR facilities 436 captured YEM were put into a 5000 L flow-through tank and left to acclimate for 1 month before commencement of the growth trials. A high survival rate of 96.1% was achieved for the fish. During the acclimation month fish were kept on a maintenance diet regime consisted of commercial fish pellets 2.3 mm (Nova ME, Skretting, Australia), 6 days week^{-1}, with three manual feeding sessions at a ration of ~2% of the estimated combined fish tank mass day^{-1}. In addition, during the acclimation month two formalin-based bath treatments (i.e. 200 ppm concentration for 1 hour) were applied to cleanse YEM of ectoparasites and any other harmful pathogens that may have been introduced to the resident tanks during their arrival from the wild. YEM capture was consented under Ministry of Primary Industries (MPI) special permit 546.

The growth experiment on YEM was initiated on 20th and 24th March 2015 and was terminated on 20th and 21st April for T1 and T2 respectively. For details about fish at the start and at the conclusion of the experiment refer to Table 2.1.

Table 2.1. Number of tagged (TAG) and untagged (NO) fish in tank 1 (T1) and tank 2 (T2) at the beginning and the end of the growth study with average mass and length values and their associated standard deviations, and stocking densities per tank.

Snapper	START		n	mean mass (g)	S.D.	mean length (mm)	S.D.	Stocking density (kg 1000 L^{-1})
	T1	TAG	75	28.6	4.7	109.5	6.2	
	T1	NO	101	31.2	6.1	112.9	7.2	1.1
	T2	TAG	76	30.2	5.7	113.7	6.6	
	T2	NO	100	29.9	6.7	111.1	7.7	1.1
	END							
	T1	TAG	43	187.9	56.2	198.3	18.7	
	T1	NO	0	N/A	N/A	N/A	N/A	1.6
	T2	TAG	24	187.8	42.6	201.4	17	
	T2	NO	0	N/A	N/A	N/A	N/A	0.9
YEM	START							
	T1	TAG	118	20.3	6.3	128.8	11.6	
	T1	NO	97	25.2	8.7	135.3	12.6	0.9
	T2	TAG	102	22.8	7.6	132.1	13.7	
	T2	NO	102	22.9	7.2	132.9	12.3	0.9
	END							
	T1	TAG	79	208.7	33.4	242.5	9.2	
	T1	NO	0	N/A	N/A	N/A	N/A	3.3
	T2	TAG	75	225.7	30.5	246.8	9.8	
	T2	NO	0	N/A	N/A	N/A	N/A	3.4

2.2.2 Tagging of experimental fish

The food unlimited growth trials started with a tagging session and taking morphometric measurements of the fish (i.e. mass and FL) for the first time. The purpose of tagging was to ensure an instant identification of subjects during the monthly measurement sessions. Approximately half of the fish per tank were tagged, and the rest were untagged (Table 2.1). The untagged fish represented the

pool of individuals intended for monthly tissue and organ sampling. When tagging, 12 mm glass coated passive integrated transponder (PIT) tags (AVID Identification systems Inc., CA, USA) were used. First, fish were anaesthetised in the tank with 25 ppm AQUI-S (Appendix 1). Approximately 30 minutes after the application of anaesthetic fish started showing signs of stage *4* anaesthesia (Appendix 1), which permitted the tagging session to commence. The abdominal skin was penetrated with the needle and a tag was injected into the peritoneal cavity. As soon as tagging was completed, a fish was scanned with the Power Tracker 2 scanner (AVID Identification systems Inc., CA, USA) for the identification number. After tagging and before placing fish in a tank with fresh sea-water (free of anaesthetics) fish mass and FL were taken and recorded.

2.2.3 Experimental set up and protocol

2.2.3.1 Weekly feeding regime and diet composition

During the food unlimited growth study both fish species were kept under the same feeding regime at all times. The weekly feeding routine had been purposely designed to sustain fish health and wellbeing. The 7-day feeding regime was composed of:

- Sunday: no feeding
- Monday: commercial aquaculture diet (4 mm pellets, Nova ME, Skretting, Australia)
- Tuesday: commercial aquaculture diet (4 mm pellets, Nova ME, Skretting, Australia)
- Wednesday: in-house formulated and prepared alginate
- Thursday: in-house formulated and prepared alginate
- Friday: in-house formulated and prepared alginate
- Saturday: commercial aquaculture diet (4 mm pellets, Nova ME, Skretting, Australia)

Ingredients in 4 mm Skretting pellets were: fish meal, animal protein, meals, plant protein meal, wheat, fish oil, poultry oil, selection of vitamins and minerals. Nutritional composition of the pellets was: crude protein 50%, crude lipid 17%, carbohydrates 17%, moisture 7%, ash 9%, total phosphorus 1.3%, gross energy 21.5 MJ, digestible energy 19.0% MJ.

Ingredients in the in-house alginate were: base (1 L distilled water, 90 g Protanal powder, 0.6 g tetra sodium pyrophosphate) and alginate [1.8 kg Fish Mince, 575 g Fishmeal, 87.5 g Minerals (salmonid premix), 70 g Vitamins (salmonid premix), 35 g Vitamin C, 3.2 g CaSO4 (Calcium sulphate), 0.172 g Astaxanthin (lucantin pink)]. Nutritional composition of the alginate according to the proximate analysis generated by Cawthron Institute (98 Halifax Street, Nelson 7042, project number:

T26325) was: crude protein 21.3%, total fat 2.7%, carbohydrate 5.6%, moisture (at 105°C) 62.7%, ash 7.7%.

2.2.3.2 Maintaining the food unlimited protocol

The unlimited food growth experiment required conditions that guaranteed fish satiation at all times, with the exception of 1 day per week when fish were not fed as the standard protocol used at PFR. To ensure that food was not the growth limiting factor two approaches were practiced during the course of the experiment. First, feed was delivered by hand multiple times per day in excess, which was verified visually by observing uneaten food at the bottom of the tank at the end of each feeding session. Fish were fed 3–4 times daily (i.e. 8:30–9:00 am, 11:30–12:00 pm, 2:30–3:00 and 5:00–5:30) from Monday to Saturday. The visual conformation that not all offered feed had been consumed was not always possible due to changes in water turbidity, therefore a second approach was also employed to support meeting the requirements of the study. The second approach was based on the feeding methods described in Booth et al. (2008), which was adapted to suit the purpose of the experiment. The first day after recovery from tagging and related anaesthesia, fish were fed 3 times with a ration of 4.5% of the estimated combined fish mass in a tank. The uneaten feed that collected on the bottom of a tank after each feed was transported by the water circulation to a mesh basket (mesh size 3 mm) attached to the outlet pipe. The following day, before the first morning feed (~8:30 am) the uneaten feed that had been gathered in the mesh basket was collected and weighed. The percentage of uneaten feed was calculated and if less than 30% of feed given the previous day was collected the next day the ration was increased by 10%. If more than 30% was collected, the next day the ration was not changed. When 50% of uneaten feed was collected the ration was decreased by 10%. Monitoring the percentage of uneaten feed was scheduled for 1–2 times $week^{-1}$. This system worked only when alginate was offered to fish since there were no means to collect pellet leftovers as they would quickly crumble and disintegrate in the water. However, uneaten alginate remains in the form it was delivered (i.e. small cubes, ~0.5–1 cm^{-3}). Furthermore, the preliminary alginate water soaking test revealed that alginate would soak sea-water equivalent to 20% of its original mass. This finding assisted in calculating true feed remains which in turn supported keeping fish at satiation throughout the study. Pellets as dry feed with much higher energetic and nutritional content relative to alginate were offered in the same quantities as alginate ensuring fish were fed to satiation independently of the type of feed provided.

2.2.3.3 Growth study measurement sessions and fish sampling for the morphometric analysis

Growth measurements (i.e. mass and FL) were taken for each fish at monthly intervals of ~30 days. The exception was snapper during winter when the frequency of sampling/measurements were reduced to approximately one sampling over a month and a half due to increased snapper health concerns through the coldest months of the year. There were in total 12 measurement sessions between October 2014 and November 2015 for snapper, and 14 sessions between March 2015 and April 2016 for YEM.

Apart from the initial measurement session, executed jointly with tagging, that signified the launch of the growth study, each consecutive measurement point included: fish sedation in tanks, scanning for identification, weighing fish mass and measuring FL. The final task required was to randomly select eight (for all YEM sessions and the first six snapper sessions) or seven (the last five snapper sessions) fish from the pool of untagged individuals for organ and tissue sampling for subsequent morphometric determination and biochemical analysis. The day before planned sampling/measurement sessions fish were not fed. That created a time window of ~40 hours, since the last feeding. This was expected to be sufficient for the guts to be fully evacuated (Booth et al., 2008). Sessions always took place in the morning ~9–10 am. First, fish were sedated with 20–25 ppm AQUI-S. After 20–30 minutes they started showing loss of equilibrium as they entered stage *3b* anaesthesia (Schoettger and Julin, 1967; Appendix 1). Anaesthetised fish were collected with a dip net, starting with those that showed the highest sensitivity to the anaesthesia (i.e. fish that started gathering at the bottom of the tank first) and put into a plastic 40 L crate (dimensions: 21 x 34 x 58 cm) containing the anaesthetised tank water. A portable working station was set up between two tanks, one from which fish were going to be measured and the second where analysed/measured fish would be released. Fish were first scanned, as described previously and tagged individuals identified. Second, all fish (tagged and untagged) were weighed on a balance, model Mettler PE 6000 (Mettler-Toledo, LLC, Columbus, OH 43240, USA), and the values were recorded. Third, the FL was taken on the 30 cm plastic measuring board (MariSource 7009 St Ct E,Fife, WA 98424, USA) to the nearest millimetre and recorded next to the corresponding identification number. And finally, in another 40 L crate also filled with the anaesthetised tank water, eight or seven randomly selected untagged fish were placed for subsequent euthanasia and laboratory work to generate morphometric and biochemical datasets (for details see chapter 5). At the end of the sampling/measurements session the recorded data were entered into an Excel spread sheet and processed.

2.2.4 Growth and morphometric data processing and parameters description and calculation

Growth and morphometric parameters within the first month (i.e. October–November 2014 and March–April 2015 for snapper and YEM respectively) were not included in the final growth analysis as this period was considered to be the "training month". The purpose of the "training month" was for the eco-physiological systems of the fish and their social structure to readjust to the fact that they were going to be kept under *ad libitum* feeding regime for the next 12 months. This training period was especially important for YEM as they had to adjust not only to enclosed captive conditions but they also had to adjust their physiological machinery to the type, frequency and quantity of feed for the duration of the experiment, as this may markedly differ to what they used to in the wild prior the capture.

2.2.4.1 Growth study

During all measurement sessions mass and length measurements were taken for all fish (i.e. tagged and untagged fish). Although data from untagged fish were not necessarily included in all parameter determinations, there were multiple reasons for their acquisition. Firstly, the data of all fish was incorporated for relative and absolute annual mass (length) gain and absolute annual mass (length) product determination. Furthermore, the information of total fish mass $tank^{-1}$ was necessary for maintenance of the feed unlimited diet regime, and it was important for calculating the percent of daily feed consumption (*DFC%*) throughout the study. Finally the untagged data served as a control to monitor for effects of tagging on growth parameters. There were no observed effects of tagging on growth performance on either of the test species.

2.2.4.1.1 Condition and growth related morphometrics

Mass–Length Relationship (MLR) within species was determined from tagged fish data with the following equation:

$$M = aL^{b},$$

Where a and b are constants (parameters), M is whole body wet mass (g) and L is FL (mm) (M and L are variables). The log base 10 ($\log_{10}$) logarithmic rearrangement of MLR:

$$log(M) = log_{10}(a) + b*log_{10}(L) + e \text{ (error term)}$$

Is in the form of a linear model thus suitable for fitting $log_{10}(M)$ and $log_{10}(L)$ to linear regression for estimating parameters a (intercept) and b (slope). The same estimation can be performed without $\log_{10}$ transformation by fitting the two parameters power curve to the mass-length data. However, transformation made the error term additive and variation within the model more uniform and stable which makes any subsequent comparison more appropriate.

Fulton's Condition Factor (*K*) is a commonly used approach to describe the condition/wellbeing of a fish. It was calculated with:

$$K = 100\frac{M}{L^3}$$

With *M* and *L* variables as described previously, the factor 100 was to bring the final value close to 1. In this study *K* was determined and reported only for completeness purposes; it is the most widely utilised condition metric for fish. *K* assumes isometric growth (Froese, 2006; Ogle, 2013), but allometric growth was prevailing for both species, especially in YEM, thus the means to express and discuss test fish condition in this study was by utilisation of relative mass in relation to mean mass.

Relative Mass in relation to mean mass (*Rm*) was the method utilised to investigate relative condition differences amongst sampling/measurement points within and across the two test species:

$$Rm(\%) = 100\frac{M}{a_m L^{b_m}}$$

Where *am* is the geometric mean of *a*, and *bm* is the arithmetic mean of *b*, across all available, mass–length estimates for a species as parameters of the mean MLR. Variables *M* and *L* are as described earlier. MLR parameters *a* and *b* were calcualted for all measurment points, taken monthly (YEM) or monthly/bimonthly (snapper) as required for the final calculation of *Rm* (Froese, 2006).

Relative Annual Mass (Length) Gain, *RAM(L)G*, expressed as:

$$RAM(L)G = 100\frac{M(L)fn - M(L)in}{M(L)in}$$

They are percentages of total annual mass and length increase, measured for all fish in tanks between first measurements after the training month and the final measurements 12 month later. *M(L)fn*

represents mean mass and length values at the termination of the growth study and *M(L)in* were mean initial mass and length values of all fish in the study.

Absolute Annual Mass (Length) Gain, *AAM(L)G* is an absolute mass and length increase measured for all fish in tanks between first measurements after the training month and the final measurements 12 month later at the termination of the experiment conducted under the *ad libitum* diet regime, expressed as:

$$AAM(L)G = M(L)fn - M(L)in$$

Where *M(L)fn* and *M(L)in* are the final mass mean and FL mean values, and initial mass mean and FL mean values respectively of all fish in the 12-month growth study.

Annual Mass (Length) Factorial Growth, *AM(L)FG* is ratio of final and initial mean mass and mean length of all fish and it tells what is the product of mass and length increased during the period of the growth experiment, calculated with following equation:

$$AAM(l) = \frac{M(L)fn}{M(L)in}$$

Where *M(L)fn* and *M(L)in* are the same as above.

Specific Mass (Length) Growth Rate (*SMGR/SLGR*), average percentage (%) of body mass and length increase day^{-1} between two sampling/measurement points, were calculated with the following equation:

$$SM(L)GR = 100\frac{lnM(L)lt - lnM(L)er}{t}$$

Where *lnM(L)lt* is a natural logarithm of body mass (g) or FL (mm) of the later measurement of any two consecutive measurement points, and *lnM(L)er* is a natural logarithm of body mass (g) or FL of the earlier measurement of any two consecutive measurement points for the individual fish, and t is number of days elapsed between the two consecutive measurement points.

Absolute Mass (Length) Growth Rate (*AMGR/ALGR*), average absolute growth increment day^{-1} achieved between two sampling/measurement points for mass and length respectively of an individual fish, were calculated with the following equation:

$$AM(L)GR = \frac{M(L)lt - M(L)er}{t}$$

Where *M(L)lt* and *M(L)er* are the same as for SMGR/SLGR except they are raw values, and *t* is the same as above.

Absolute Cumulative Mass (Length) Gain (*ACMG*/*ACLG*), absolute cumulative growth increments of individual fish expressed as mean mass and mean length values obtained during the all consecutive measurement sessions (training month not included) for the extend of the growth experiment.

Daily percent of feed consumption (*DFC*) per estimated total fish mass tank^{-1}:
DFC calculation was based on measurements of feed remains as explained in 2.2.3.2. First by use of *SMGR*, the total fish mass was estimated for the day before which the uneaten feed was collected. *DFC* was calculated with the following equation:

$$DFC(\%) = 100\frac{Mef}{Mtf}$$

Where *Mef* is total mass of eaten feed on the day before feed remains collection, *Mtf* is the estimated total fish mass in the tank on the day before feed remains collection.

2.2.4.1.2 Organosomatic indices and supplementary morphometrics

Body indices of interest were: relative gutted mass (gutted mass index – GMI), hepato-somatic (HSI), cardio-somatic (CSI), spleno-somatic (SSI), entero-somatic (ESI) and visceral (mesenteric) lipid (VLI) index.

Body indices, as a percentage gutted, organs and visceral fat mass of the total body mass were generated with the following equation:

$$Body\ Indices\ (\%\ body\ mass) = 100\frac{OM}{TM}$$

Where *OM* represents mass of inner organs (i.e. heart, liver, spleen and pyloric caeca with intestines), gutted fish mass and total visceral fat mass; while *TM* is total body mass of the given fish taken after euthanasia.

2.2.5 Creation of arbitrary seasonal groups according to sea-water temperature oscillation

To assisting interpretation of data, a calendar year was arbitrarily arranged according to the intensity of sea-water temperature oscillation in the Nelson Haven into four time periods named as long summer, short autumn, long winter and short spring. Sea-water temperature from December to April (4-month period) on average is relatively stable oscillating about 3°C, ~18.5–21.5°C. Similarly, seawater temperature between June and October varies from ~9.5 to 12.5°C. Therefore, those two 4-month sections were called long summer and long winter, and note that the terminology does not match entirely with the common use of the terms summer and winter. Conversely, periods from April to June and October to December (2-month periods) are characterised with a relatively rapid drop or rise (~6°C) in sea-water temperature from ~18.5 to 12.5 and from ~12.5 to 18.5°C respectively. Following the same logic as above, those two periods were named short autumn and short spring.

2.2.6 Statistical methods

2.2.6.1 General overview

For all statistical procedures data from both tanks were pooled together and wherever permissible, *tank* as a factor was included in the model as a random effect or blocking factor to account for extra variation due to any differences in tanks. Furthermore, days between two consecutive sampling/measurement points were not always the same for both tanks within species. Therefore the number of days between the two measurement points were averaged for both tanks wherever the numbers of days were not the same for the given period.

The statistical analyses in the thesis were carried out in Microsoft Windows Excel (2013), SigmaPlot version 12.5 (Systat Software, Inc., San Jose California USA, www.systatsoftware.com) and R, *free software* for *statistical* computing and graphics (www.r-project.org).

Following any sampling/measurement session, data were entered into an Excel spread sheet where initial graphical investigation, curve fitting and descriptive statistics were performed. Excel was also utilised for correlation tests and to connect the time series data via the curve smoothening polynomial function. All through the study 4th polynomial order was employed as a standard, unless stated differently. The core purpose of utilising polynomial function as a curve smoothing method was to assist revealing any patterns associated with seasonal temperature change that may appear too

cryptic to be detected (graphically or statistically) on the basis of the raw data only. Therefore, a correlation test in search for an association between raw temperature data and the predicted values generated from the 4th polynomial equation fitted to the raw response variable (e.g. *SMGR* or *HSI*) was practiced. Nevertheless, whenever the coefficient of determination R^2 associated with 4th polynomial function was > 0.90 only raw dataset was used for the correlation test, because it was expected that in those cases smoothening function would not markedly improve data fit. However, in a few cases when the R^2 was greater than 0.90 predicted data did show fit improvement by producing p-value below significance level of 0.05 which was not the case with the raw data, indicating presence of a possible trend which would otherwise remained obscured. Therefore, in the result section, both correlation outputs from predicted and raw data were presented where applicable.

In several cases the *lag time* correlation pattern (the pattern between two variables that becomes statistically significant when data of one variable are moved for one or a few data points left or right) was observed. When such a pattern appeared to be present, prior to correlating data set with sea-water temperature, data in question were moved for a few time points and compared with mean monthly temperature rather than the raw temperatures measured on days of the sampling. Since the same tagged fish (Table 2.1) were repeatedly used as subjects of the growth experiment on which multiple mass and FL measurements were taken (i.e. 11 and 13 for snapper and YEM respectively), the appropriate statistical model to account for breaching assumption of independency was a mixed effects model based on repeated measures (RM) ANOVA. RM ANOVA was performed in SigmaPlot with the fixed effect being time (or measurement points) and the random effects being fish (as subjects) and tanks. The rest of the mandatory assumptions (i.e. normality and homoscedasticity) were automatically tested in SigmaPlot before performing statistical calculations. In SigmaPlot a Shapiro-Wilk normality test was employed to test for normality of residuals, and homoscedasticity or equality of variances was verified by comparing absolute values of residuals against fitted values with a linear regression model where p-value < 0.05 rejected the null hypothesis demonstrating violation of the assumption. Any violation was firstly required to fit the model with the transformed data (i.e. methods of transformation used: Log10, square root or reciprocal –response variable^{-1}). Surprisingly, in all SGRs and AGRs cases for both species, assumptions of the model were violated and no transformation procedures improved the model fit, hence the utilisation of RM ANOVA on ranks was warranted and performed. When differences between groups (i.e. measurement points) were detected either Tuckey HSD Pairwise multiple comparisons of means or Holm-Sidak pairwise multiple comparison procedures were offered by SigmaPlot and performed. When it was required to test the effects of one or two factors on multiple groups one-way ANOVA (analysis of variance) or two-way ANOVA was employed respectively. ANOVA was performed in R, and assumptions were tested after the test statistics manually. The assumption of normality was checked graphically by constructing a normal probability plot of residuals. In ANOVA, only when the assumption of normality was markedly violated

transformation of a response variable was warranted. This is because ANOVA is fairly robust to violation of this assumption. However, special care was taken when violation of homoscedasticity or equality of variances occurred, which was checked manually but in the same way as explained for RM ANOVA in SigmaPlot. The process after assumption testing was the same as for RM ANOVA. When transformations did not fix violation of assumptions a non-parametric Kruskal-Wallis ANOVA on Ranks was employed. Detected differences between groups were identified with a post-hoc Tuckey HSD Pairwise test.

Relationship testing via the linear regression model were also performed in SigmaPlot. The assumption testing and transformation were the same as for ANOVA.

A Student's t-test was employed where only two groups were being compared. The assumptions of t-test were verified in the same manner in SigmaPlot as for ANOVA and regression; and when violation occurred the same transformations as explained earlier were employed. Finally, when the non-parametric test was endorsed, a Mann-Whitney Rank Sum Test was carried out. R software was specifically utilised where certain manipulation of the model was required which could not be performed via SigmaPlot. In this chapter certain rearrangements of the linear regression model were required, thus they were processed in R.

Significant difference between two concepts or groups was considered when the significance probability value $P \leq 0.05$. All data throughout the thesis were presented either as means (circle symbols) with 95% confidence limited error bars, or when a relationship between two variables was not portrayed with the curve of the best fit or variables are not compared between the species, data were graphed in box-plot format (see chapter 4, 5 and 6), unless otherwise stated.

2.2.6.2 MLR

- Was determined by employment of the linear regression model once MLR equation was logarithmically rearranged.

- To investigate if there was a difference between MLR between two species, comparison of parameters from the log transformed regression model $log_{10}\ (a)$ and b was performed. For comparison purposes a factor *species* as a new variable was required to be added to form a new extended version of the original regression model. Subsequently the significant

interaction term between log_{10} *(L)* and factor *species* would indicate significant differences in MLR between species. The analysis was performed in R.

2.2.6.3 Condition Factors

- *Rm* was calculated from tagged fish growth data and compared between species with Student's t-test. RM ANOVA was employed to test for differences amongst measurement points. Rm was also correlated with temperature data. All statistical procedures followed descriptions outlined in the general statistical overview above.

2.2.6.4 Growth rates

- SGRs, AGRs and ACGs were tested with RM ANOVA for differences amongst measurement points.

- Effects of the annual temperature oscillation on the growth rates, and also *DFC*, was tested by the correlation test against corresponding temperatures as both, predicted values via the 4th polynomial curve fitting and as raw data.

2.2.6.5 Supplementary morphometrics

- Body indices – differences amongst sampling points were tested with two-way ANOVA, with a main factor time (i.e. sampling points, months, throughout the period of one calendar year) and tank as a blocking factor.

- Effects of the annual temperature oscillation was tested in the same way as growth rates by employing 4th order polynomial function and correlation test.

2.3 Results

2.3.1 Mass – Length Relationship (MLR)

2.3.1.1 Snapper

The MLR of snapper is portrayed graphically in Figure 2.4A (in an arithmetic form explained with the two-parameter power function) and Figure 2.4B (in a logarithmically rearranged form). The exponential MLR (M = 0.000006 $L^{3.257}$) states that the mass of snapper exposed to annual food unlimited conditions is increasing exponentially with the increase in unit of length with the exponent b of 3.257, or the same argument expressed in base-10 logarithmic form with the equation of the best fit (R^2 = 0.98) $\log_{10}$ (M) = -5.211 + 3.257 *$\log_{10}$ (L) with 95% confidence limits for a = -5.289 – -5.132, and b = 3.222 – 3.292. The results indicated that snapper for the period of study exhibited overall allometric growth ($p < 0.001$) as specified with the range of confidence limit which does not include b exponent value of 3.

2.3.1.1.1 Seasonal MLR

To examine any changes in MLR during the 12-month study, MLR was calculated separately for different seasons. The data were grouped into arbitrarily designated seasons based on magnitude of seawater temperature oscillations. The MLRs for the four seasons in their linear forms with 95% confidence limits for parameters a and b and with the type of growth (allometric or isometric) are outlined in Table 2.2. Even though snapper, when all growth data from the study were examined together, exhibited allometric growth, when seasonal MLR was calculated an isometric type of growth during winter and spring was revealed (Table 2.2). Furthermore, when seasonal MLRs, analysed with linear regression model, were compared amongst each other, it was evident that the only group that statistically differed from other was the long summer group. This was indicated with significant interaction between $\log_{10}$ (L) and factor *season* (summer-autumn, $F_{1, 428} = 14.25$, $p < 0.001$; summer-winter, $F_{1, 428} = 21.65$, $p < 0.001$; summer-spring, $F_{1, 428} = 24.19$, $p < 0.001$). The positive direction of all four regression lines with the results obtained from regression model comparisons indicate that the summer fish on the log10 transformed data increase in a unit of length, increased their mass significantly more than fish from any other seasons.

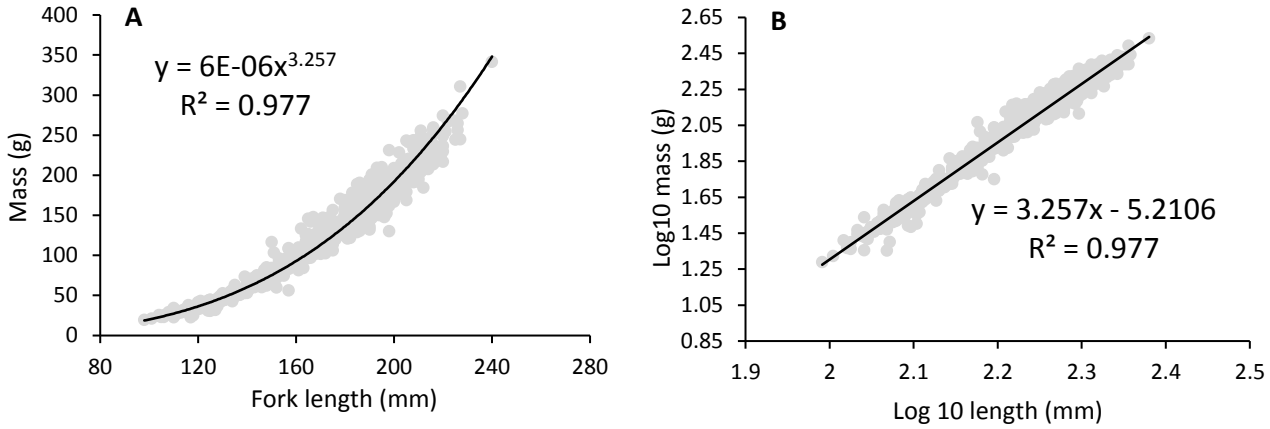

Figure 2.4. Exponential (A) and log-10 base transformed (B) mass-length relationship data of snapper for the duration of the growth experiment from November 2014 to November 2015, with associated equations and coefficient of determination (R^2).

2.3.1.2 YEM

Figure 2.5 (A) and (B) show an arithmetic and logarithmic model of YEM MLR respectively, for the course of the growth study. According to the models the relationship can be expressed as: mass of YEM for the duration of growth experiment increases exponentially for any increase of unit length with the exponent *b* of 3.364 ($M = 0.000002\ L^{3.364}$) and in the $\log_{10}$ transformed arrangement: *log(M)* = -13.112 + 3.364 **log(L)* with a tight data fit to the model with $R^2 = 0.98$ and confidence limits: a = -5.745 – -5.644, b = 3.342 – 3.386. The same as for snapper, the range of *b* confidence intervals indicate allometric growth of YEM when all data from the 12-month study were combined.

2.3.1.2.1 Seasonal MLR

Unlike snapper, YEM exhibited positive allometric growth even when annual data were divided amongst the four arbitrarily selected seasons (Table 2.2). In similar fashion as the summer snapper, only YEM regression MLR models from the first 2 months of the growth study (i.e. April and May 2015) representing a short autumn differed from all other seasons. However, the difference was not as strong as for snapper (autumn–winter, $F_{1,\ 817} = 4.68$, $p = 0.030$; autumn–spring, $F_{1,\ 544} = 4.18$, $p = 0.041$; autumn–summer, $F_{1,\ 821} = 13.04$, $p < 0.001$). The same analogy as for snapper can be applied

when interpreting YEM MLR findings. Therefore, the autumn growth log_{10} transformed YEM data implies that as fish length increased, their mass increased significantly more than for any other season.

Table 2.2. Snapper and YEM log-10 base transformed mass-length relationships for four arbitrarily arranged seasons with 95 % confidence intervals for parameters *a* and *b* with associated R^2 and types of growth.

Species	Season	Log10 transformed MLR	*a*	*b*	R^2	Growth type
			95% confidence limits			
Snapper	long summer	log (M) = -5.628 + 3.450*log (L)	-5.788 – -5.468	3.376 – 3.524	0.97	allometric
	short autumn	log (M) = -4.906 + 3.135*log (L)	-5.170 – -4.642	3.019 – 3.252	0.95	allometric
	long winter	log (M) = -4.707 + 3.030*log (L)	-5.012 – -4.402	**2.896 – 3.164**	0.93	**isometric**
	short spring	log (M) = -4.669 + 3.011*log (L)	-4.984 – -4.353	**2.873 – 3.149**	0.93	**isometric**
YEM	short autumn	log (M) = -6.133 + 3.553*log (L)	-6.375 – -5.891	3.441 – 3.666	0.94	allometric
	long winter	log (M) = -5.817 + 3.427*log (L)	-5.952 – -5.681	3.365 – 3.488	0.97	allometric
	short spring	log (M) = -5.709 + 3.376*log (L)	-5.990 – -5.428	3.253 – 3.500	0.91	allometric
	long summer	log (M) = -5.525 + 3.288*log (L)	-5.751 – -5.299	3.192 – 3.385	0.89	allometric

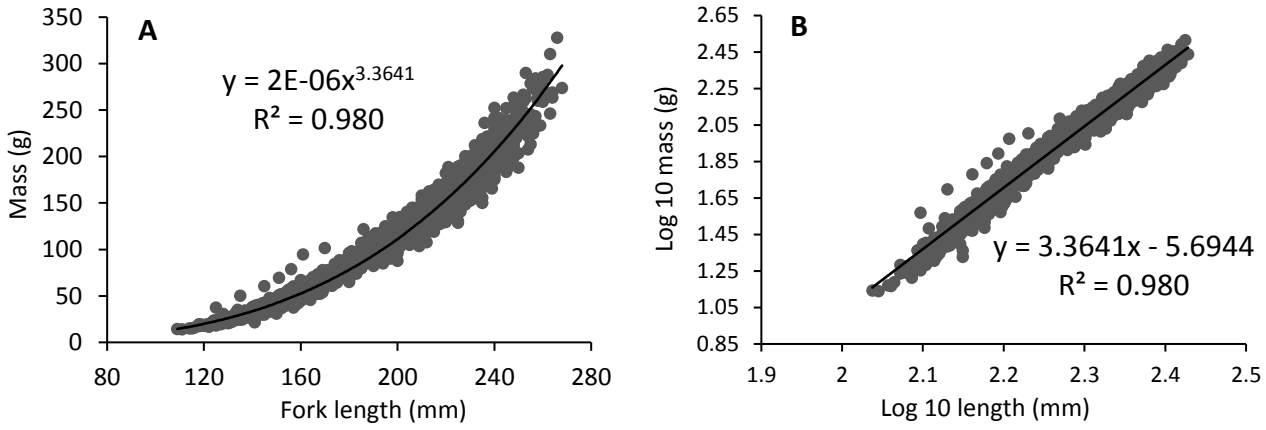

Figure 2.5. Exponential (A) and log-10 base transformed (B) mass-length relationship data of YEM for the duration of the growth experiment from April 2015 to April 2016, with associated equations and R^2.

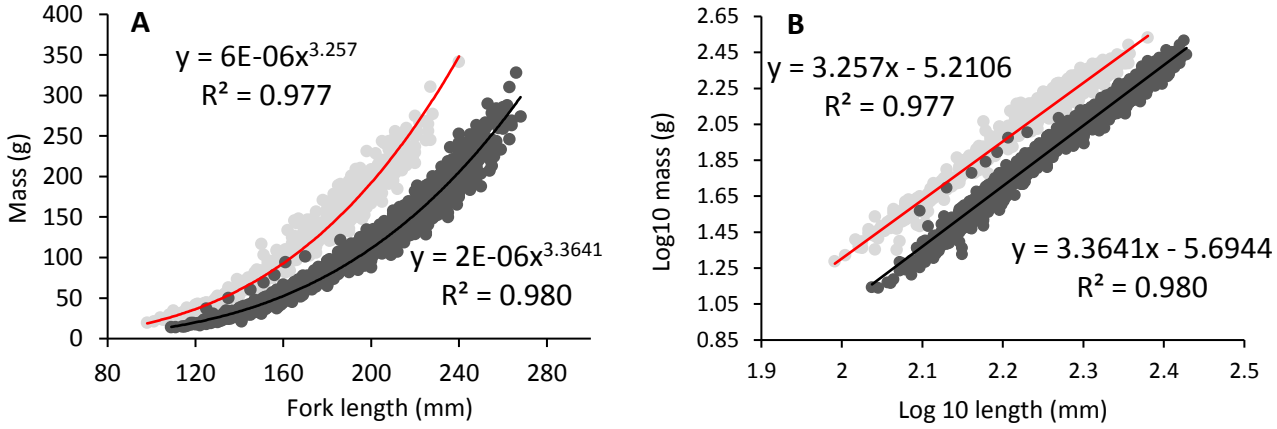

Figure 2.6. A – snapper (light grey symbols and red curve) and YEM (dark grey symbols and black curve) raw mass-length relationship data for the period of 12-month growth study fitted to power model (curves) with associated equations and R^2s. B – log-10 base transformed mass-length relationship data for snapper (light grey symbols and red line) and YEM (dark grey symbols and black line) from Figure A, fitted to linear model with associated equations and R^2s (black lines).

2.3.1.3 MLR – snapper vs. YEM

During 1 year of intense growth snapper and YEM demonstrated different characteristics of MLR ($F_{1,\ 2582} = 26.18$, $p < 0.001$). The MLR trajectories on the logarithmically transformed data (Fig. 2.6B) supported by statistically confirmed differences indicated that YEM on average increased its mass for any unit of length more than snapper. That is not apparent when the raw data is graphically presented (Fig. 2.6A). The reason for this apparent discrepancy lies in the different intercepts or starting mass-length point of the two curves and the fact that snapper MLR has a lower *b* exponent (i.e. 3.257) than YEM (i.e. 3.364). This is accounted for with the log transformation.

2.3.2 Fish condition: Fulton's Condition Factor (*K*) and Relative Mass (*Rm*)

2.3.2.1 Snapper

Fulton's condition factor (*K*) and relative mass in relation to mean mass (*Rm*) for snapper are portrayed against annual temperature curve in Figure 2.7 and 2.8 respectively. Generally, there are no marked differences between the two, since as mentioned earlier, snapper showed overall allometric but seasonally both allometric and isometric growth. Nevertheless, *Rm* was utilised to compare the relative condition amongst sampling points (months) within and between the two test species, since YEM demonstrated strong allometric growth all through the course of the experiment. *Rm* of snapper changed throughout the 12-month growth experiment (Friedman Repeated Measures ANOVA on Ranks, $\chi^2 = 473.16$, df = 10, $p < 0.001$). The change followed the temperature oscillation curve (the red curve in Fig. 2.8) with approximately 2–3 months' lag time. As expected, a marked increase in condition occurred at the turn of the year 2014/15 during the summer when seawater temperature started reaching 20°C (Fig. 2.8). The increase in condition in linear fashion followed for approximately 4 months until sea-water temperature dropped below 17°C at the end of April 2015. With a further decrease in sea-water temperature during the course of late autumn and winter, fish condition was also in the declining phase. When the temperature started rising by the end of the winter - early spring, the increase in *Rm* gradually started to be evident again.

A correlation test between *Rm* and temperature data further statistically confirmed a strong underlying association between the two data sets (Table 2.4). However, a direct correlation test did not suggest existence of any relations between *Rm* and temperature. Only when *Rm* dataset was taken for three data points backwards as suggested by the lag time in the graphical illustration the connection between temperature and *Rm* was revealed as a strong positive correlation (Table 2.4).

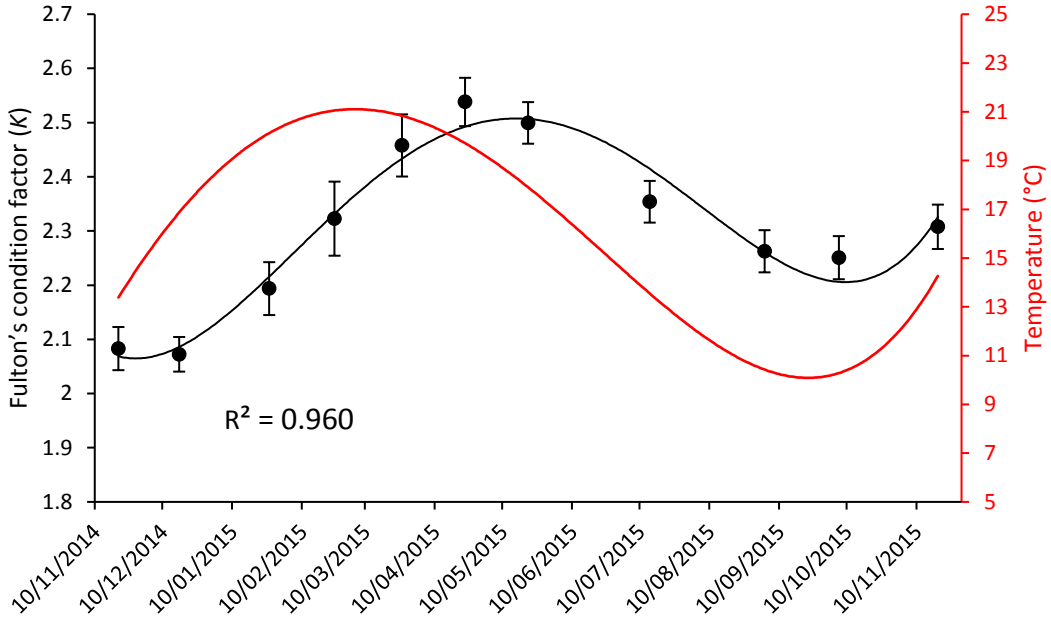

Figure 2.7. Fulton's condition factor (*K*) of snapper (black symbols with associated black curve) through the 12-month growth study superimposed with the temperature curve (red). Both curves were derived from raw data fitted to 4th polynomial function (temperature data points omitted for clarity). R^2 belongs to the 4th polynomial function fitted to the *K* data. Error bars are 95% confidence intervals. The caption of other graphs in the chapter are equivalent to Fig. 2.6, unless stated differently.

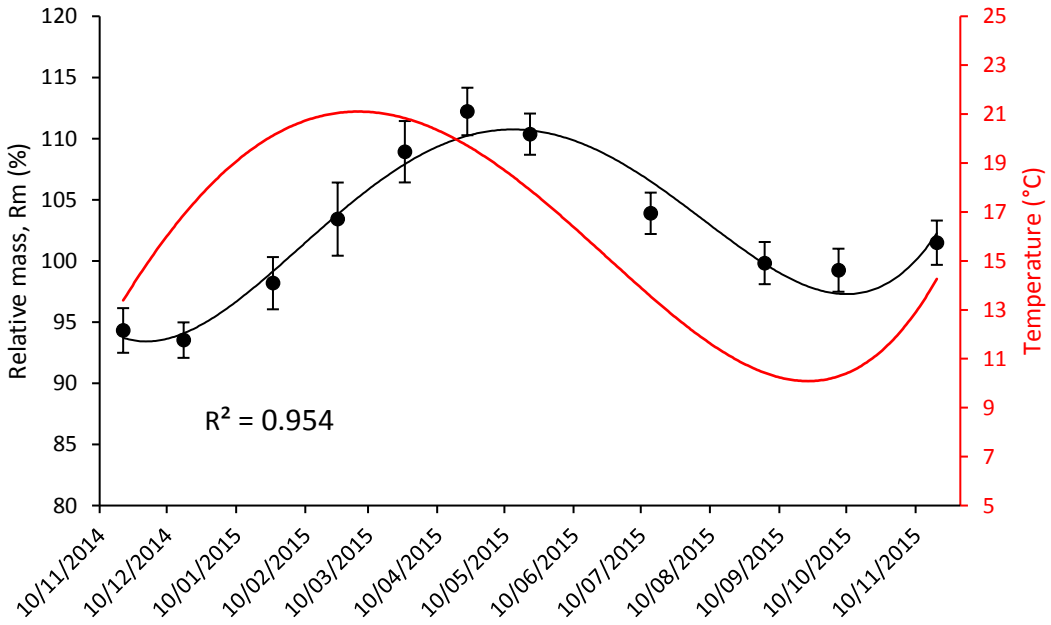

Figure 2.8. Relative mass in relation to mean mass (*Rm*) of snapper.

2.3.2.2 YEM

In the same manner as for snapper, *K* (Fig. 2.9) is reported for the completeness of data presentation, as well as to illustrate a potential error that could be introduced if *K* is the only means to explain YEM (or any other fish exhibiting allometric growth) condition.

Rm differed amongst sampling/measurement points of the growth experiment (ANOVA on Ranks, $\chi^2 = 750.82$, df = 12, $p < 0.001$; Fig 2.10). Initially, unlimited food conditions supported a hasty jump in YEM *Rm* in autumn. The rate of the increase gradually slowed down with the start of and throughout winter. During spring time *Rm* exhibited a decrease that continued at a slow rate until February, characterised with a yearly peak sea-water temperature of ~ 21°C. At that period *Rm* stabilised and finally again started to rise in late summer and early autumn (Fig. 2.10).

A correlation test used to investigate the strength of relation between *Rm* and seawater temperature demonstrated a moderately strong, significant negative correlation (Table 2.4).

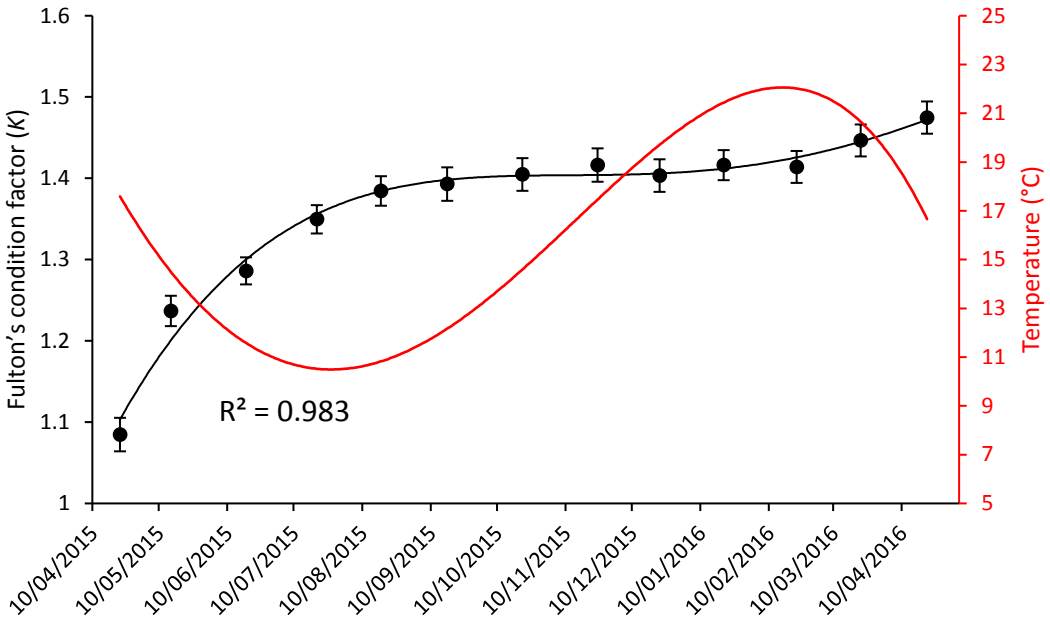

Figure 2.9. Fulton's condition factor (K) of YEM.

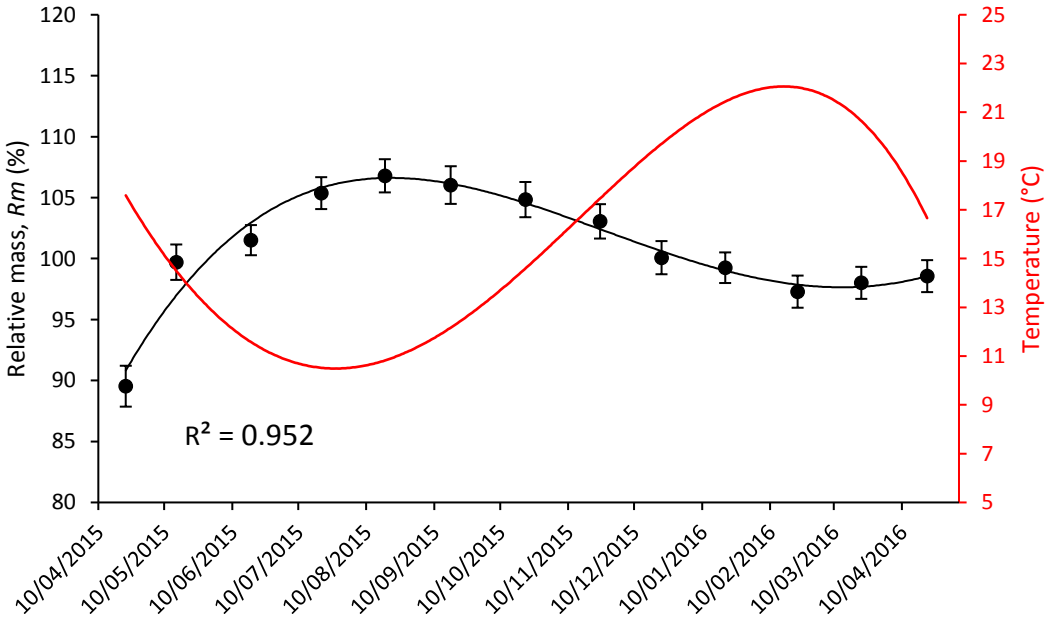

Figure 2.10. Relative mass in relation to mean mass (*Rm*) of YEM.

2.3.2.3 Condition – snapper vs. YEM

Rm is an appropriate matrix to compare condition across different populations and species (Froese, 2006). Average annual snapper *Rm* (mean = 102.3 ± 6.2 S.D., n = 11) was only negligibly higher than YEM's (mean = 100.8 ± 4.8 S.D., n = 13) as confirmed with Student t-test which did not show significant deviation between the two *Rm*s. However, the *Rm* responses showed different overall dynamics in terms of how they correlated with temperature, which in turn were similar relative to their starting points. They both began with a strong increase until the plateau was reached within the first 5 or 6 months in the experiment for YEM and snapper respectively (Fig. 2.11). Afterwards a decrease of *Rm* was noted; however, YEM *Rm* did not decline at such a high rate as snapper, and again both had a similar ending with the last 3 points indicating another turn in dynamic from decline to increase (Fig. 2.11).

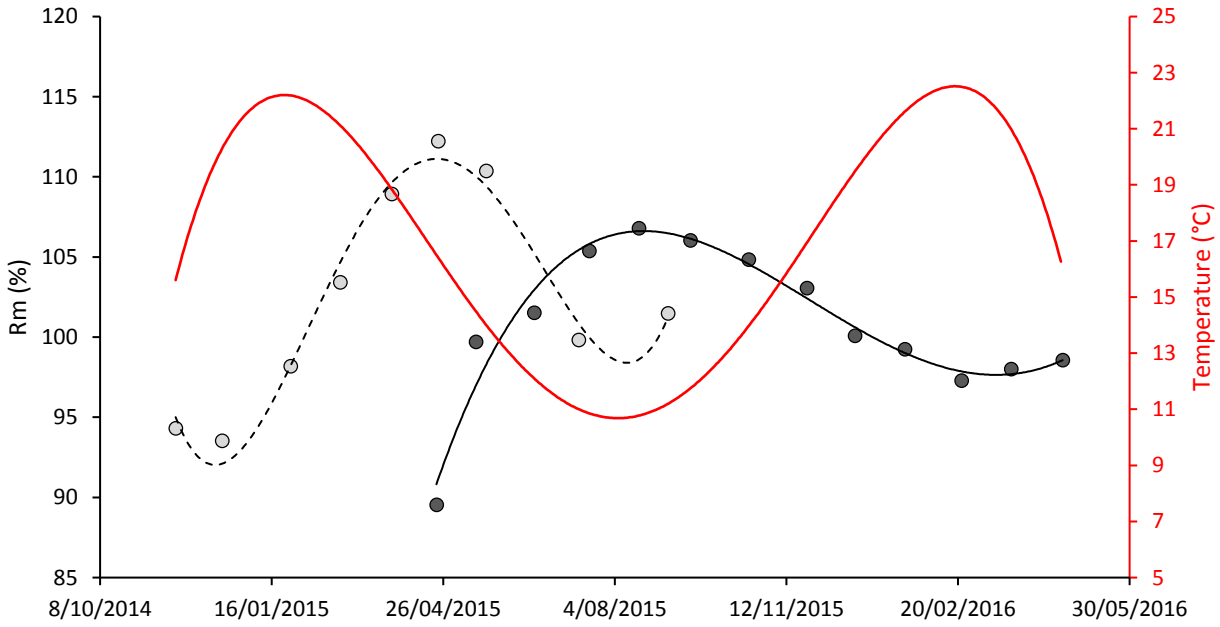

Figure 2.11. Relative mass (*Rm*) response of snapper (black dashed curve and light grey symbols) and YEM (black curve and dark grey symbols) on the time scale encompassing actual times of the growth experiment of both species and superimposed with the temperature curve (red). All curves were derived from raw data fitted to 4th polynomial function.

2.3.3 Growth descriptive statistics, Relative Annual Mass (Length) Gain [*M(L)G*] and Absolute Annual Mass (Length) Gain [*AM(L)*], Annual Mass (Length) Factorial Growth [*AM(L)FG*]

2.3.3.1 Snapper and YEM

A summary of initial and final mean values of growth variables with their associated standard deviations and ratios for both species is outlined in Table 2.3. Snapper initial mean mass was similar to but still statistically different from YEM initial mass (Mann-Whitney U Statistics on ranks = 44735.50, $p < 0.001$) whose variability was 33 % higher indicated by its standard deviation (Table 2.3). These descriptive statistics turned to be in reverse by the end of the trials where final YEM mean mass was higher than snapper's (U test statistics = 3859.50, $p < 0.001$) yielding a final average mass increase (AMFG) of 6.81 times of its original mass compared with snapper whose mass increased 5.26 times. Furthermore, the mass variability within the snapper sample increased 6.1 times while YEM's increased only 2.6 times, reaching only about half of snapper final mass variability. Original, as well as concluding length values also differed between the two species, both being higher for YEM (U statistics = 12933.00, $p < 0.001$ and U stats = 78.50, $p < 0.001$ for the beginning and the end length values respectively), with similar factorial growth (Table 2.3). In terms of length variability, the same smaller to higher (snapper) and higher to smaller (YEM) pattern was observed. Overall YEM was more "successful" in growth achievements since all annual growth parameters YEM/snapper ratios were above 1 (Table 2.3).

Table 2.3. Descriptive statistics of initial and final growth variables and related relative and absolute annual calculations based on measurements of all fish (i.e. tagged and untagged fish) with standard deviations (S.D.), sample sizes and associated ratios.

	Snapper			YEM			Mean ratio	S.D. ratio
	mean	S.D.	n	mean	S.D.	n	SN/YEM	SN/YEM
Initial M	35.4	8.34	312	31.83	12.37	392	1.11	0.67
Final M	186.2	50.87	81	216.59	32.68	154	0.86	1.56
Initial L	118.93	9.07	312	140.32	14.89	392	0.85	0.61
Final L	199.01	17.74	81	244.6	9.74	154	0.81	1.82

Mass	RAMG (%)	AAMG (g)	AMFG	RAMG (%)	AAMG (g)	AMFG
	426.01	150.8	5.26	580.56	184.76	6.81
Length	RALG (%)	AALG (mm)	ALFG	RALG (%)	AALG (mm)	ALFG
	67.33	80.07	1.67	74.31	104.27	1.74

	RAMG (%)	AAMG	AMFG	RALG (%)	AALG	ALFG
YEM/SN	1.36	1.23	1.29	1.1	1.3	1.04

Legend*: *M – mass, L – length, SN – snapper, RAMG – Relative Annual Mass Gain, RALG – Relative Annual Length Gain, AAMG – Absolute Annual Mass Gain, AALG – Absolute Annual Length Gain, AMFG – Annual Mass Factorial Growth, ALFG – Annual Length Factorial Growth.

2.3.4 Specific growth rates and their association with sea-water temperature

2.3.4.1 Snapper

2.3.4.1.1 Specific Mass (SMGR) and Length (SLGR) Growth Rate – or relative (%) daily mass gain calculated for the period between the two measurement points

Unlike previously described snapper condition (i.e. *K* and *Rm*), SMGR more strictly followed the temperature through the experimental period (Fig. 2.12). Moreover, the predicted 4th polynomial SMGR curve is a mirror image of the temperature curve that strongly positively correlated (Table 2.4). SMGR started moderately at a mean of 0.46 % that almost tripled within 2 months climaxing at 1.18% corresponding with the peak of the seawater temperature (~22°C) for the summer. High SMGR was maintained for several months until a rapid decline was observed between April and May 2015 concurrently with the temperature dropping below 18°C. SMGR continued declining and became negative by the middle of winter and stayed at that level until it changed its direction upward with spring time again as sea-water temperature increased (Fig. 2.12).

A similar trajectory was observed for SLGR (Fig. 2.13). The only notable difference was that during the winter period average SLGR did not exhibit length loss at any time even though it was not uncommon to observe individual fish with zero or even negative length growth. Similarly, to SMGR, SLGR was highly correlated with sea-water temperature (Table 2.4).

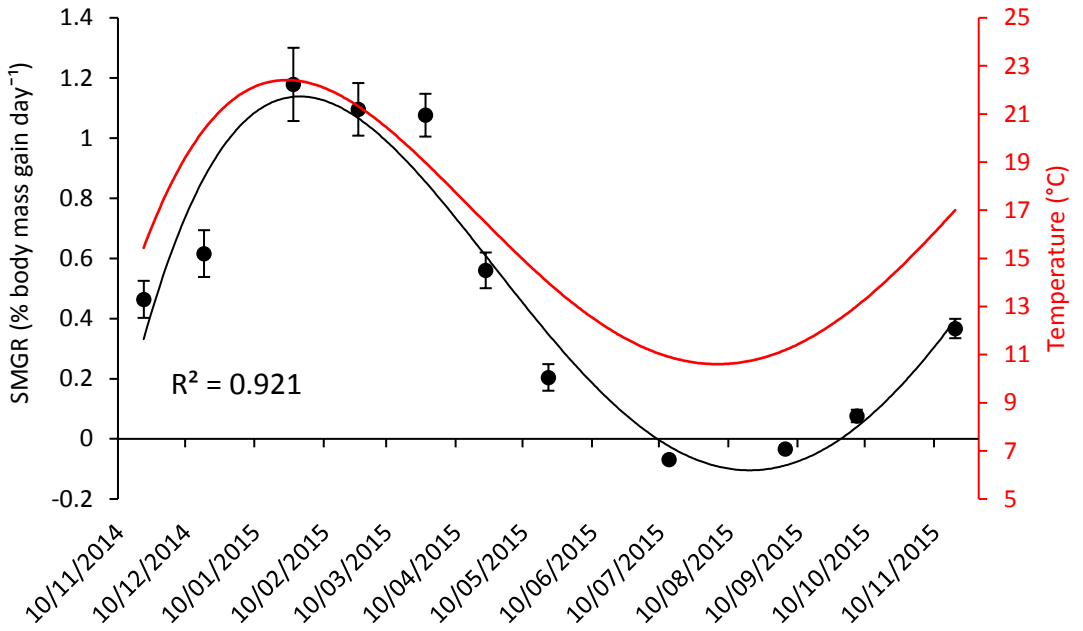

Figure 2.12. Specific mass growth rate (SMGR) of snapper.

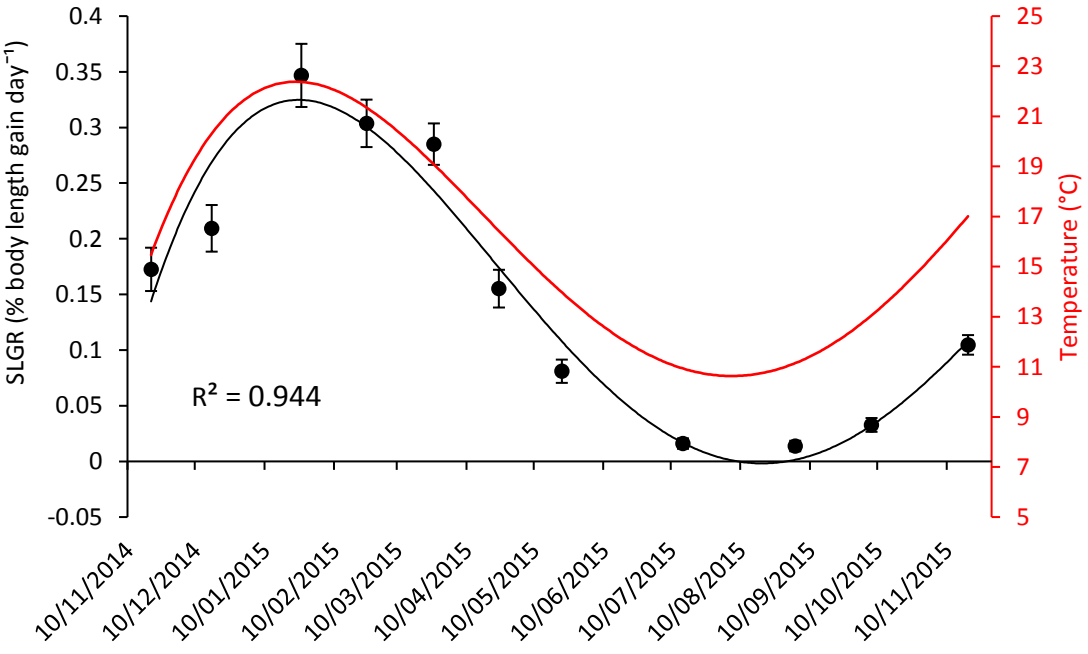

Figure 2.13. Specific length growth rate (SLGR) of snapper.

2.3.4.1.1.1 Maximum growth rates and temperature

In this study maximum specific growth rates were attained at the highest sea-water temperature for both mass and length values and conversely the lowest rates were observed at the lowest temperature. This was in general agreement with overall strong correlation between specific growth rates and temperature (R = 0.961). However, the general linear model was not the best to explain the character of the relationship, but rather 2nd polynomial function as depicted in the Fig. 2.14.

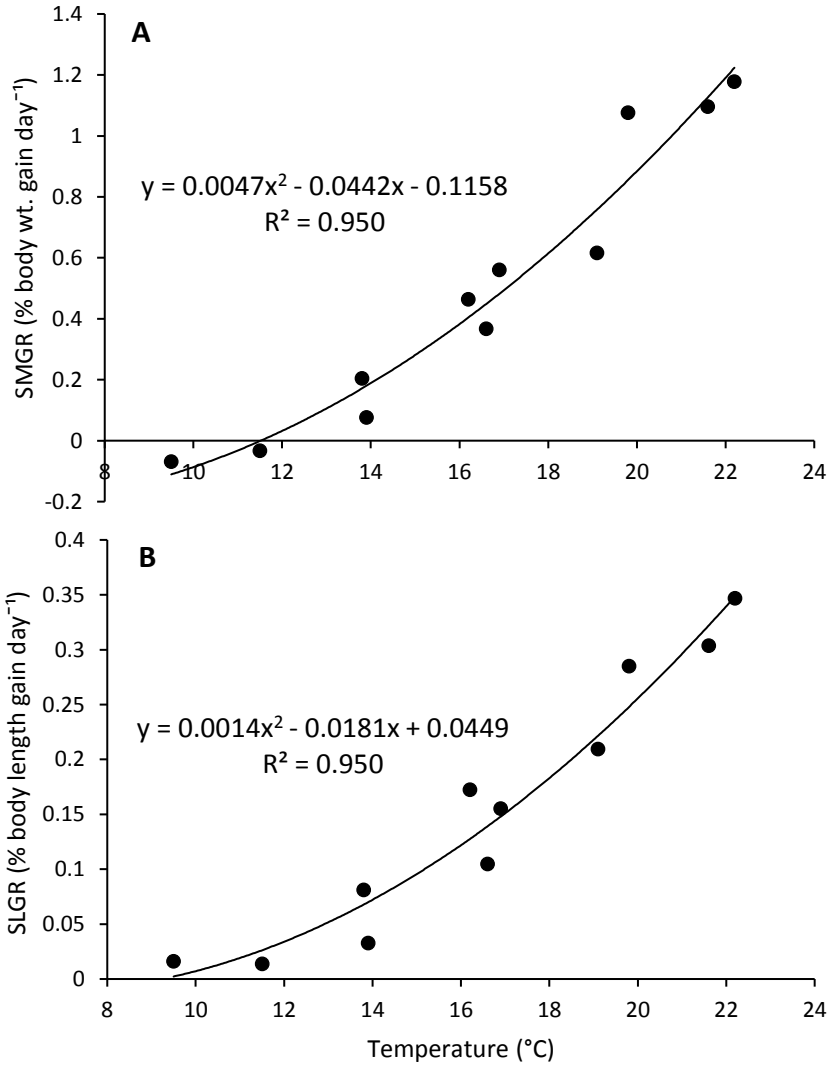

Figure 2.14. Scatter plot of snapper SMGR (A) and SLGR (B) as function of temperature for the 12-month growth study. Black curves are 2nd polynomial function fitted to the data with associated equations and R²s.

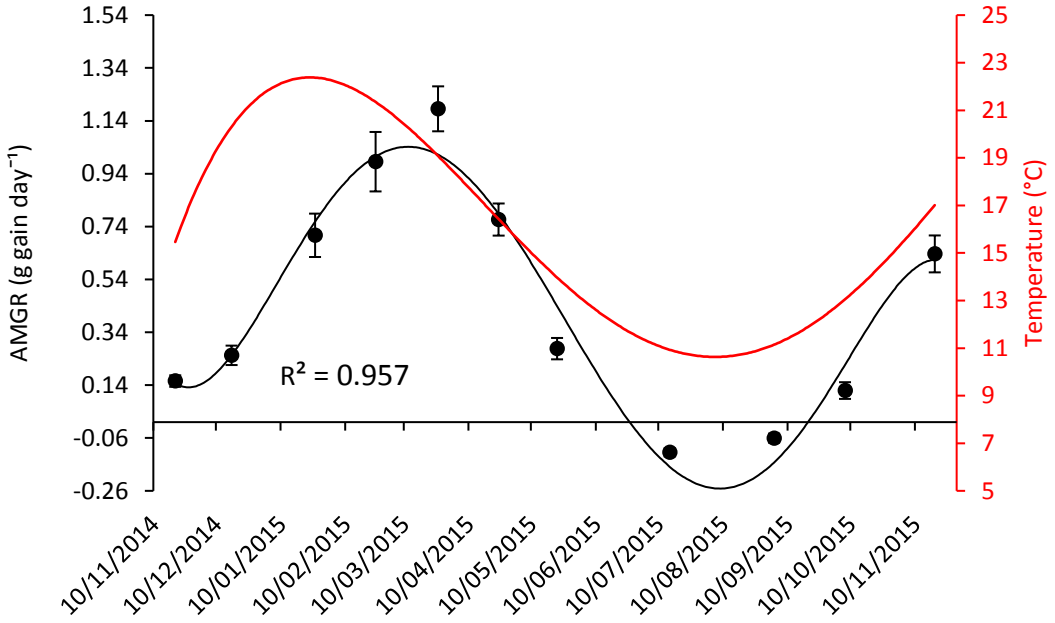

Figure 2.15. Absolute mass growth rate (AMGR) of snapper. AMGR curve was derived from raw data fitted to 5th polynomial function, which was used to highlight AMGR trajectory offset from strictly following the temperature curve. R^2 belongs to the 5th polynomial function fitted to the AMGR data.

2.3.4.1.2 Absolute Mass (AMGR) and Length Growth Rate (ALGR)

As expected, snapper AMGR overall behaved similarly to SMGR with the only obvious difference noted for the period after December 2014 when AMGR increased throughout summer in linear fashion until it reached its maximum (1.19 g day^{-1}) by the end of March 2015 (Fig. 2.15), whereas SMGR summer plateau was reached earlier (i.e. January 2015). A steep decline to the lowest AMGR point (-0.11 g day^{-1}) in the middle of the winter followed (Fig. 2.15). Even though the peak of AMGR was somewhat offset from the strict mirror match to the course of the temperature curve, strong correlation was observed for both predicted and raw data sets when regressed against temperature dataset (Table 2.4).

ALGR data well resembles SLGR in its annual trajectory ($R = 0.978$, $R^2 = 0.956$, $F_{1, 10} = 195.17$, $p < 0.001$), therefore an aforementioned SLGR description can be also applied for ALGR (Fig. 2.16).

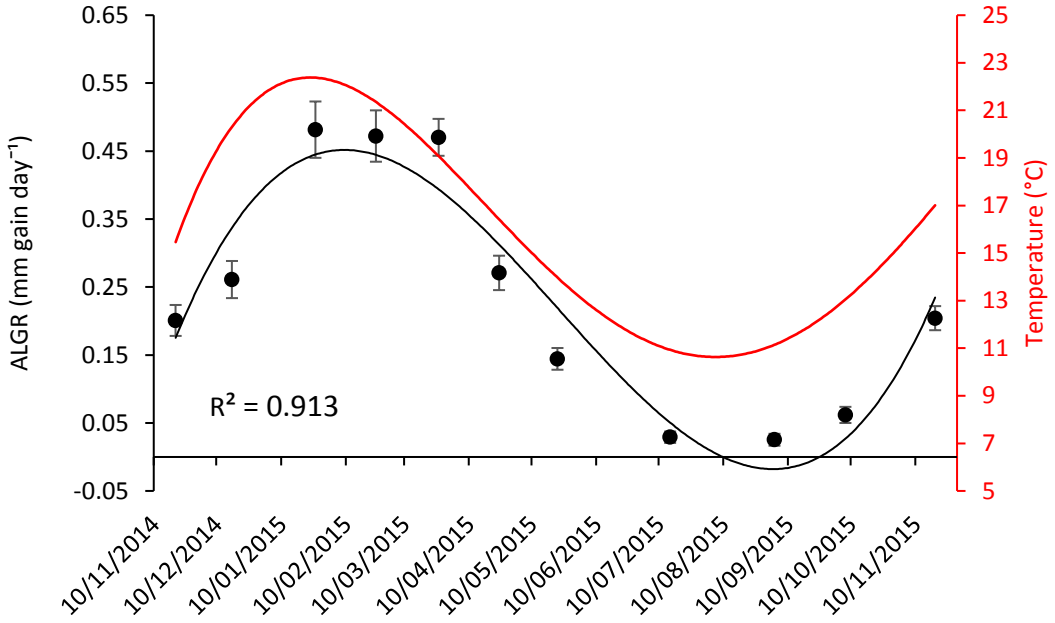

Figure 2.16. Absolute length growth rate (ALGR) of snapper.

2.3.4.1.3 Absolute Cumulative Mass (*ACMG*) and Absolute Cumulative Length Gain (*ACLG*)

Since the official start of the growth study coincided with the beginning of summer, characterised with optimal environmental conditions for growth, snapper for the first 4 months exhibited exponential mass increase as described by the equation: $M = 4E\text{-}194e^{0.0107*DAYS}$, $R^2 = 0.990$. The growth rate slowed down in early autumn when there was a decline of ambient sea water temperature to below 18°C. On average, by the end of May 2015 the snapper mass growth completely ceased. The mass increase did not resume for approximately four months until mid-spring which coincided with increase in sea-water temperature above 17°C (Fig. 2.17).

To investigate how sea-water temperature relates with ACMG a correlation test was undertaken. Direct correlation between two data sets showed non-significant, moderate correlation with the negative direction. However, with the same approach to move ACMG dataset forward or backward alongside the temperature curve as employed for *Rm*, correlation between ACMG and temperature became evident. When ACMG dataset was moved one data point (month) forward on the time scale and regressed against the average monthly temperatures a significant, negative, moderate to strong correlation was detected (Table 2.4).

ACLG had a highly similar annual trajectory as ACMG (Fig. 2.18), therefore everything mentioned for ACMG also applies for ACLG. Exponential growth within the first 4 months can be expressed with the equation: $1E\text{-}53e^{0.003*DAYS}$, $R^2 = 0.993$.

Generally, for snapper absolute growth rates revealed for both growth variables a stepwise like growth trajectory. That is illustrated with the exponential character of growth within the initial 4–5 months, following virtually no growth for the next four months. The process seemed to be recommencing again with the late spring mirroring the very start of the growth study (Fig. 2.17, 2.18).

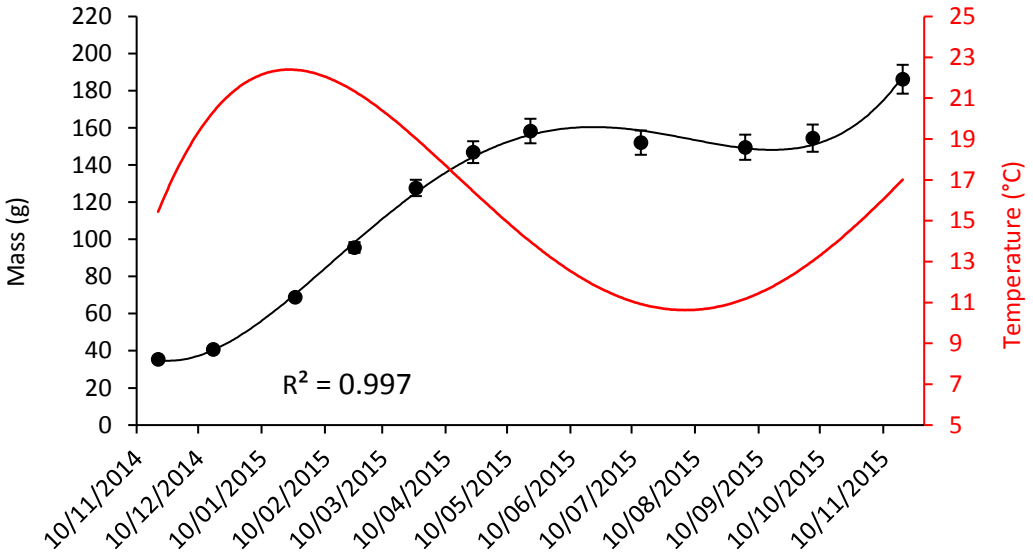

Figure 2.17. Absolute Cumulative Mass Gain (ACMG) of snapper.

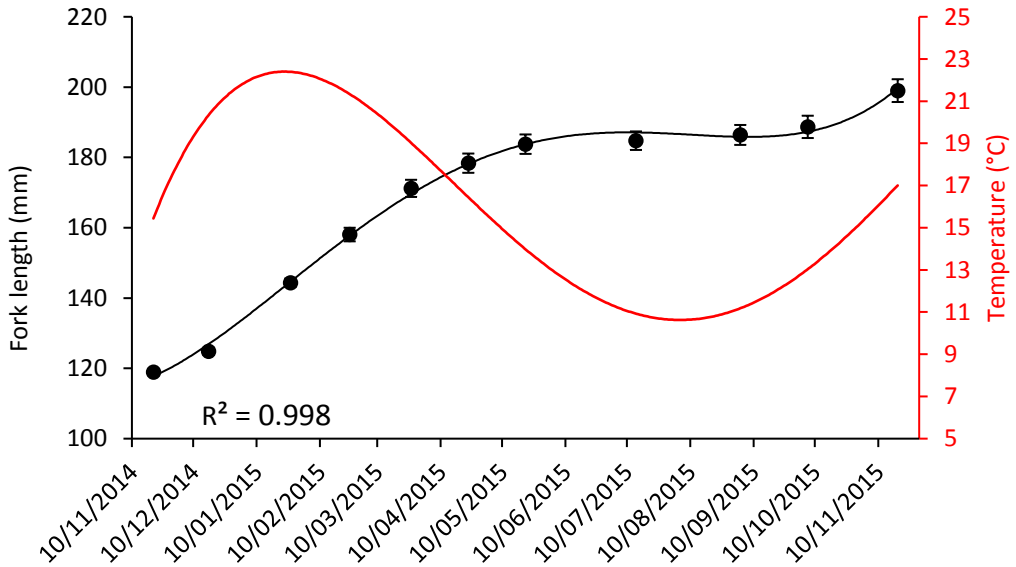

Figure 2.18. Absolute Cumulative Length Gain (ACLG) of snapper.

2.3.4.2 YEM

2.3.4.2.1 Specific Mass (SMGR) and Length (SLGR) Growth Rate

YEM straight after the acclimation period exhibited a very high SMGR, which in turn further increased during the first experimental month to 1.40 % body mass gain day^{-1} in mid-May. This made the initial growth period, in terms of relative body mass gain per day, ~2.5 times greater than the average of the all other data obtained over the course of the 12 experimental months (Fig. 2.19). After the initial extremes SMGR declined rapidly in June and continued decreasing with a slower rate until it reached the lowest levels in mid-September. After reaching its bottom extreme SMGR started increasing for the following 2 months to change its direction again with a gradual drop observed for the next 3 consecutive months with the final levels being somewhat maintained until the end of the experiment. This overall SMGR trajectory did not show significant correlation with the sea-water temperature.

Since mass and length relationship was one of the ongoing features depicting the growth character of the fish under scrutiny, SLGR was predictably similar in nature to SMGR (Fig. 2.20). However, SLGR did not vary as substantially (i.e. ~1.5 times) as SMGR between the initial few months and the rest of the study data.

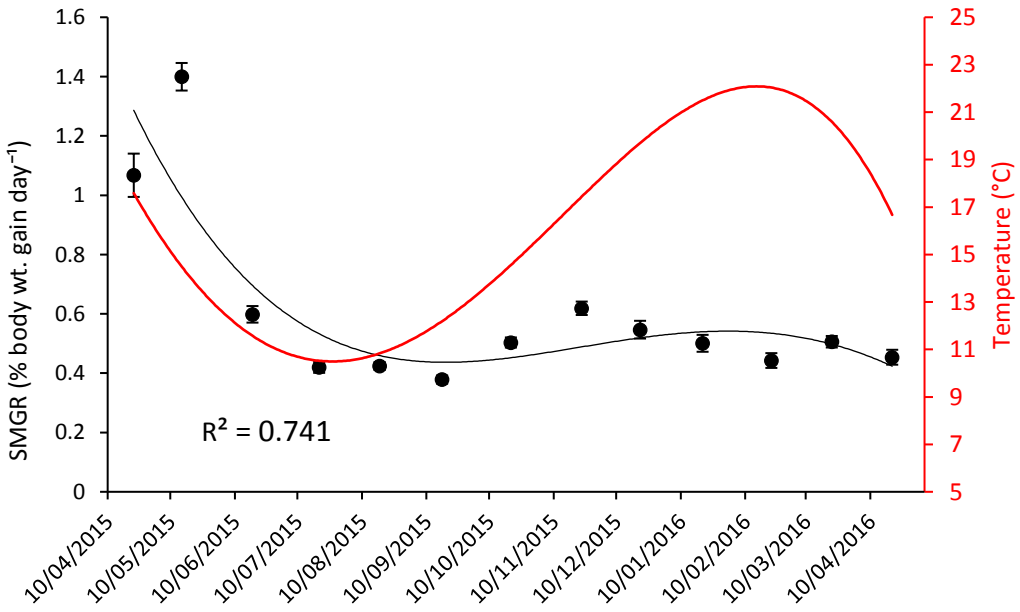

Figure 2.19. Specific mass growth rate (SMGR) of YEM.

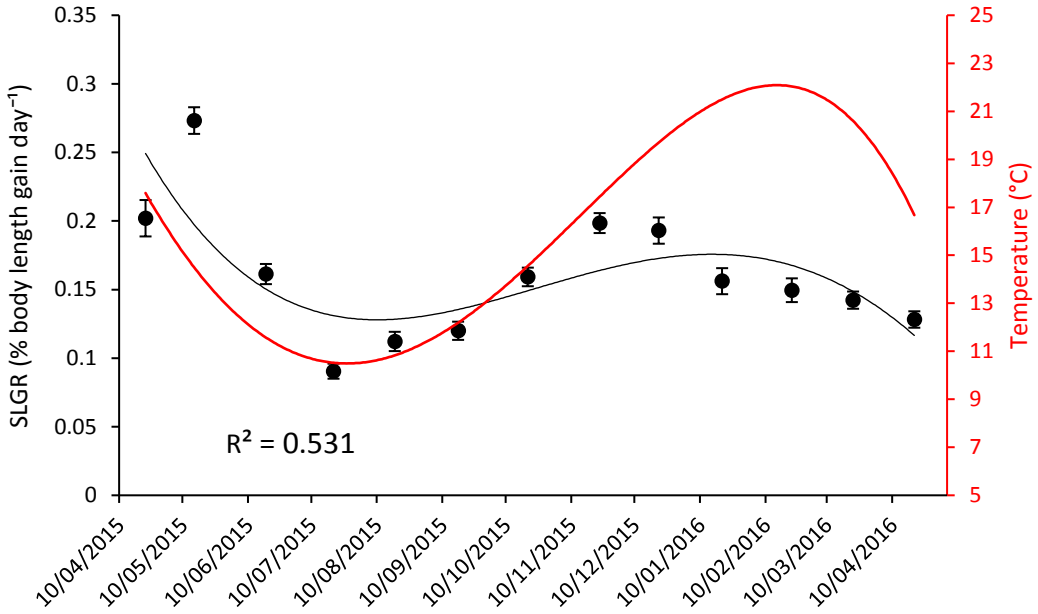

Figure 2.20. Specific length growth rate (SLGR) of YEM.

2.3.4.2.1.1 Maximum growth rates and temperature

Contrary to snapper, it appeared that YEM through the 12-month growth experiment experienced a temperature range where it was possible to observe a period during which temperature was positively, and a period when it was negatively, correlated with growth. Therefore, the transition point between the correlation directions (positive and negative) signified a temperature when the growth was most sustained (Fig. 2.21). Maximum growth rates were found at 17.2 and 18.0°C for SMGR (Fig. 2.21A) and SLGR (Fig. 2.21B) respectively. However, the first 2 months data points of the growth rates/temperature relationship were omitted from the analysis, since they were found to be outliers for the case of SMGR (see Fig. 2.19) when examined with extreme studentized deviate (ESD) method. SLGR data points during the early period in the study could not be classified as outliers (see Fig. 2.20); nevertheless, they were still removed for the analysis to be aligned with SMGR, and because YEM generally appeared not to fully adjust to the satiation feeding regime for the first 2 months in the study. This could be assumed since a year later the rates were markedly lower although at the similar temperatures. The best model to fit the data was 2nd polynomial function. Nevertheless, temperature explained only 21% variation in SMGR and 48% variation in SLGR, which may imply that mass growth was less dependent on temperature relative to growth in length.

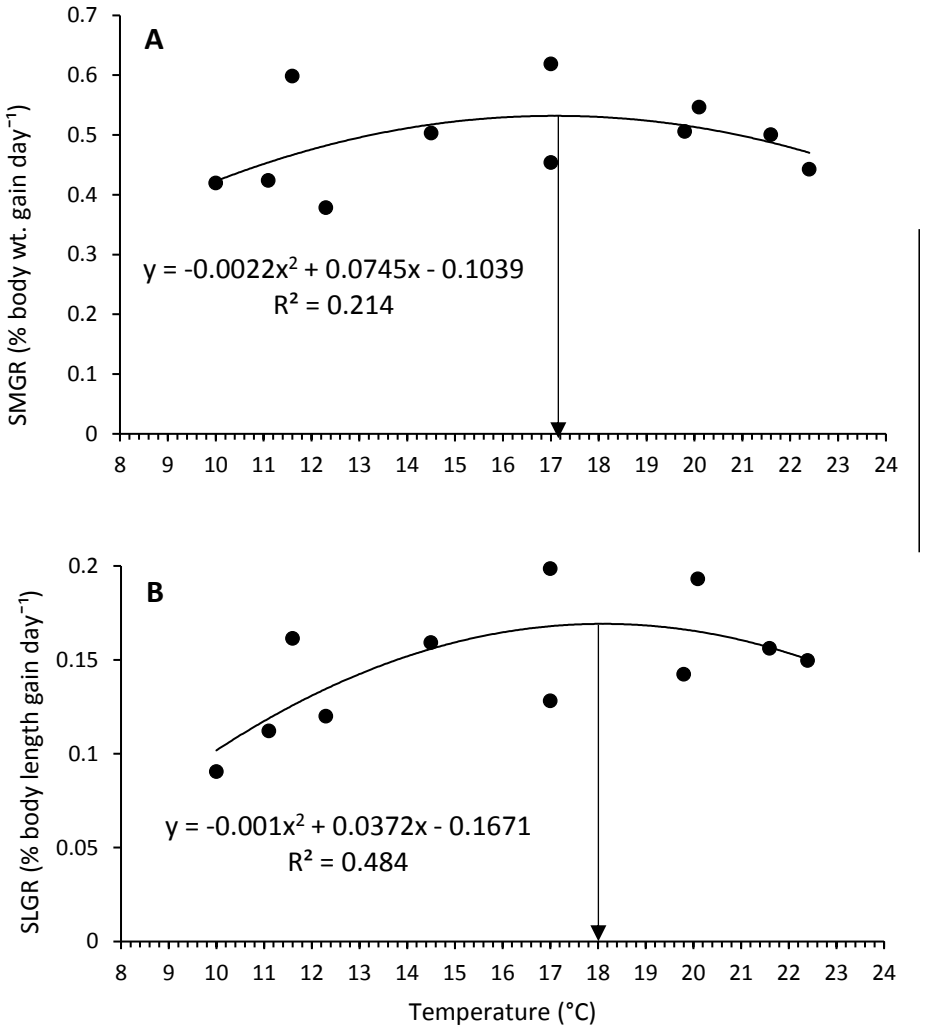

Figure 2.21. Scatter plot of YEM SMGR (A) and SLGR (B) as function of temperature for the 12-month growth study. Black curves are 2nd polynomial function fitted to the data with associated regression equations and R²s. Black vertical arrows connect the highest point on the curves as the highest growth rate with the respective temperature, where in A highest rate was at 17.2°C and in B at 18.0°C. Note that first two data points representing first 2 months in the study are excluded (refer to Fig. 18 and 19; for explanation see text).

2.3.4.2.2 Absolute Mass (AMGR) and Length Growth rates (ALGR)

YEM AMGR increased markedly from April to May 2015 then dropped to starting levels in June where it remained for the duration of winter (Fig. 2.22). With the beginning of sea-water temperature rise after the winter period, AMGR was increasing until late November and it was maintained at that level for the next 3 months with only a minor increasing trend. The last 2 months were characterised with the highest AMGR levels. The overall dynamics of AMGR, unlike specific growth rates, was positively correlated with sea-water temperatures for both raw and predicted data (Table 2.4).

YEM ALGR, similarly to AMGR, started with a rapid increase from April to May following 2 months of steep decline, to be at its lowest level in August. Then for the rest of the experiment ALGR followed the trajectory of the sea-water temperature curve (Fig. 2.23). Overall ALGR's course fairly well resembled the yearly temperature fluctuation therefore a very strong, positive correlation was observed for the two datasets (Table 2.4).

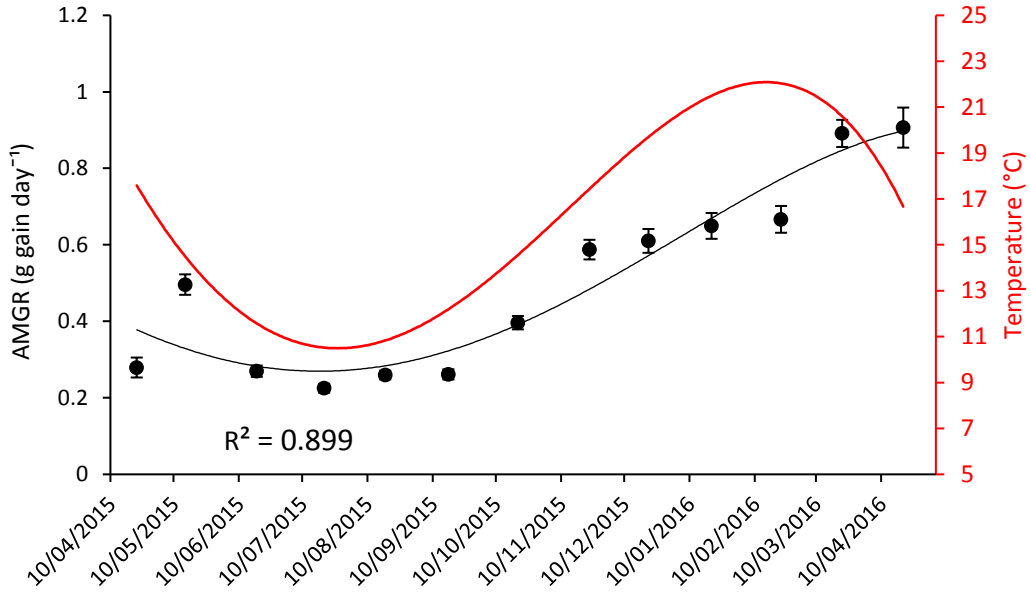

Figure 2.22. Absolute mass growth rate (AMGR) of YEM.

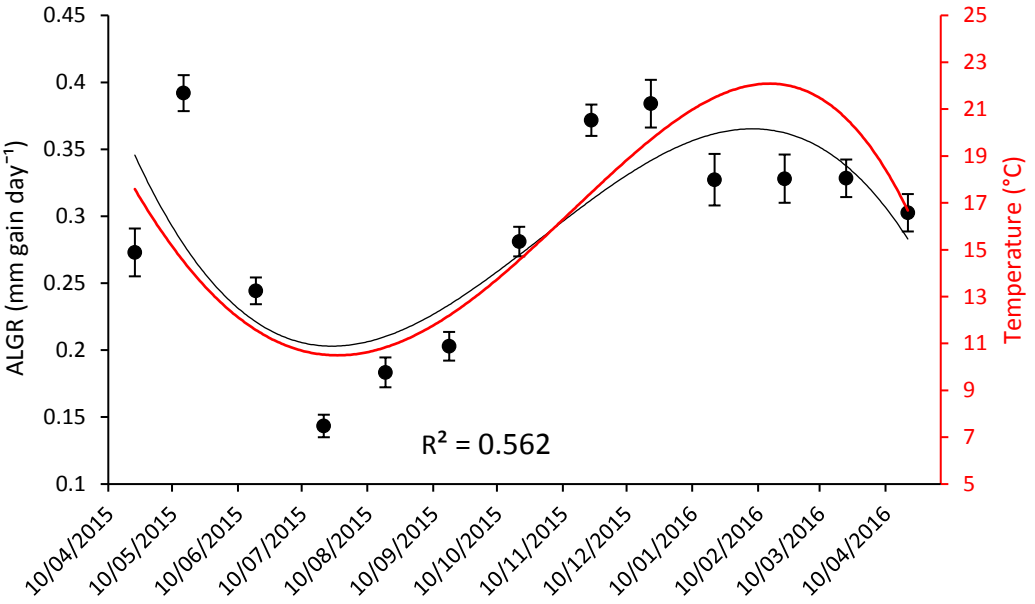

Figure 2.23. Absolute length growth rate (ALGR) of YEM.

2.3.4.2.3 Absolute Cumulative Mass (*ACMG*) and Absolute Cumulative Length Gain (*ACLG*)

YEM ACMG was expected to be well explained by the linear model. That was certainly the case in this study where the model explained 95.5% of the data (Fig. 2.24). However, the better fit was generated when 4th polynomial function was employed to explain the YEM absolute cumulative growth (R^2 = 0.999). The slight difference between the two models, points to the time in the calendar year when growth slowed down during the winter season. When a more detailed investigation was carried out comparing predicted slopes produced by first 2 months of the study representing autumn and the 4 following months representing the long winter, a difference between the two regression lines was observed ($p = 0.049$, $F_{1,\ 3} = 10.15$). Note, that since the regression model is not permissible with only two data pairs, for the purpose of comparing autumn versa winter slopes, the training month data was utilised so that the number of data pairs representing autumn was three. The character of this data point worked in favour of the null hypothesis that stated that there was no difference between the two slopes, therefore any results conveying a significance level of 0.05 and below further support acceptance of the alternative hypothesis that there was a difference. The differences between autumn (characterised with a decline in seawater temperature from ~20 to 15°C) and winter (when the temperature was maintained at ~10–11°C as the lowest in a year) suggests temperature effects on YEM growth, albeit, less so compared with snapper. In addition, SMGR and especially AMGR during the winter period exhibited the lowest values, which further supports the notion of temperature playing a role in winter growth.

Association between ACMG and sea-water temperature was further sustained by the fact of their significant positive correlation (Table 2.4). The significance of the test was based on the greater middle portion of the data representing the second half of winter, spring and first half of summer (sea-water temperature increasing), while during autumn (March–May, the period when sea-water temperature was declining) data points (curves) of the two variables have an opposite direction indicating negative correlation.

YEM ACLG is almost a perfect reflection of AMGR therefore all principles described for ACLG apply also for ACLG (Fig. 2.25). The linear model does a marginally better job in explaining ACLG data (98.8 %) than ACMG, whereas 4th polynomial function was similarly highly efficient with 99.9% of data explained by the model. The difference between regression slopes, signifying short autumn and long winter, was even more emphasised for ACMG ($p = 0.016$, $F_{1,\ 3} = 30.77$).

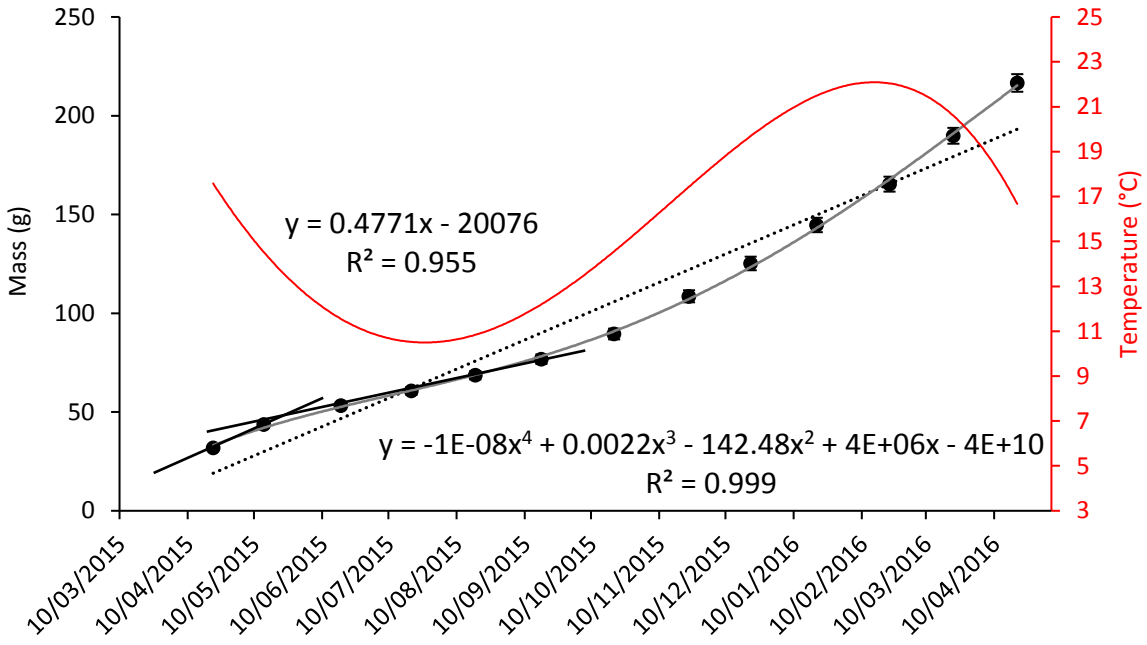

Figure 2.24. Absolute Cumulative Mass Gain (ACMG) of YEM (grey curve and black symbols). The black dotted line with the equation and R^2 (above) belong to linear regression explaining YEM growth data. The equation with R^2 (below) represents 4th polynomial function fitted to the ACMG data. Short and long black lines associated with the ACMG data represent regressions for autumn and long winter respectively.

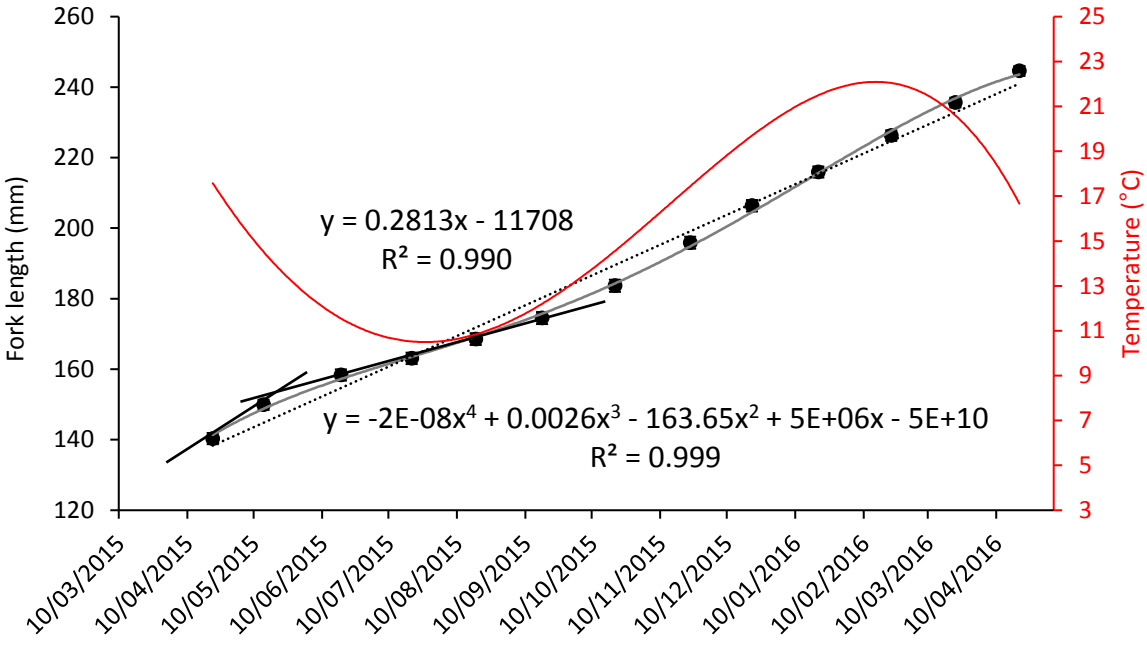

Figure 2.25. Absolute Cumulative Length Gain (ACLG) of YEM (grey curve and black symbols). The rest of the Figure caption is the same as for Fig. 2.24.

2.3.4.3 Growth Rates – snapper vs. YEM

2.3.4.3.1 SMGR and SLGR

Average yearly SMGR and SLGR did not differ statistically between the two species for the 12-month experimental period. However, when specific growth rates of test species were graphically displayed on the time scale together with the sea-water temperature curve some systematic differences become apparent (Fig. 2.26, 2.27). Snapper behaviour was strictly related and well explained with the trajectory of the temperature curve, whereas temperature concordance with YEM data was apparent only for approximately the first 7 seven months. Overall, Snapper and YEM behaved similarly through the period of autumn, winter and spring. During summer snapper continued following the course of temperature. At the same time YEM reduced SGRs and even showed a tendency to be negatively correlated with the summer sea-water temperature (Fig. 2.26, 2.27). Another difference between the two species was in the amplitude (i.e. difference between the highest and lowest values in the dataset) where snapper exhibits ~25 and ~80% higher amplitudes for SMGR and SLGR respectively.

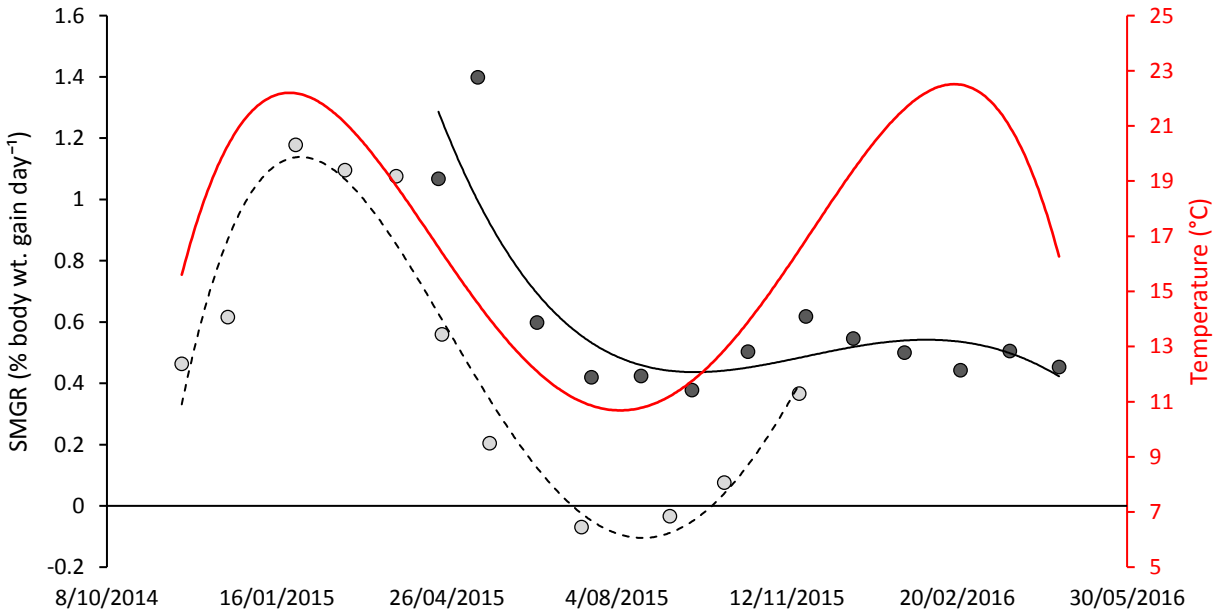

Figure 2.26. Specific mass growth rate (SMGR) of snapper (black dashed curve and light grey symbols) and YEM (black curve and dark grey symbols). The rest of the Figure caption is the same as for Fig. 2.11.

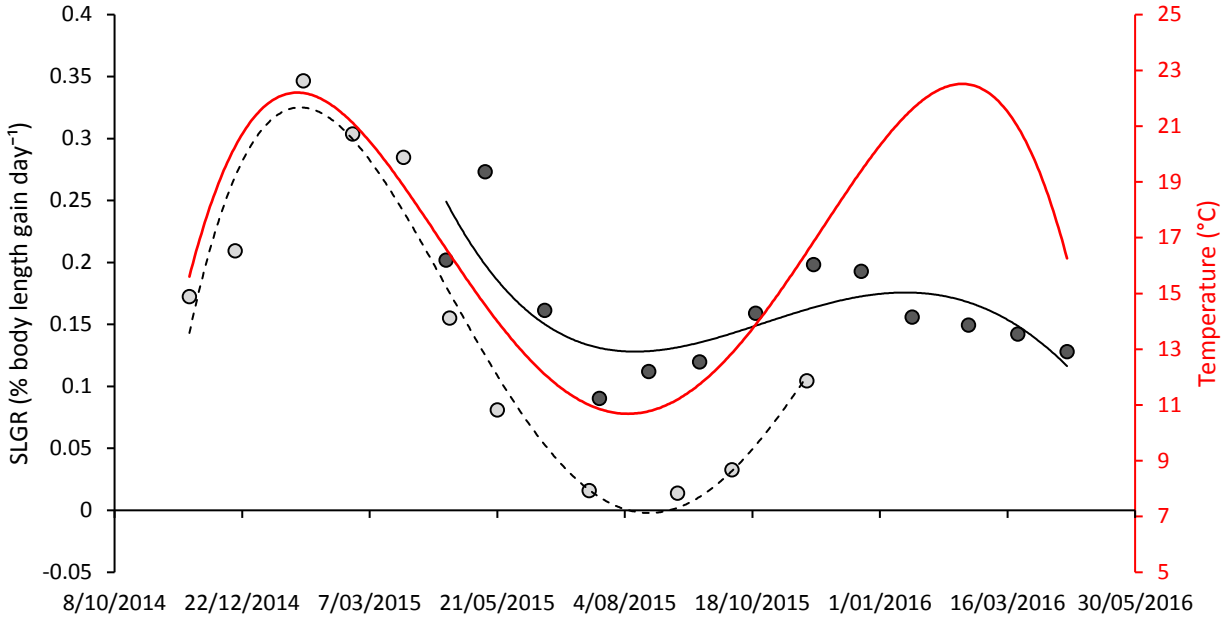

Figure 2.27. Specific length growth rate (SLGR) of snapper (black dashed curve and light grey symbols) and YEM (black curve and dark grey symbols). The rest of the Figure caption is the same as for Fig. 2.11.

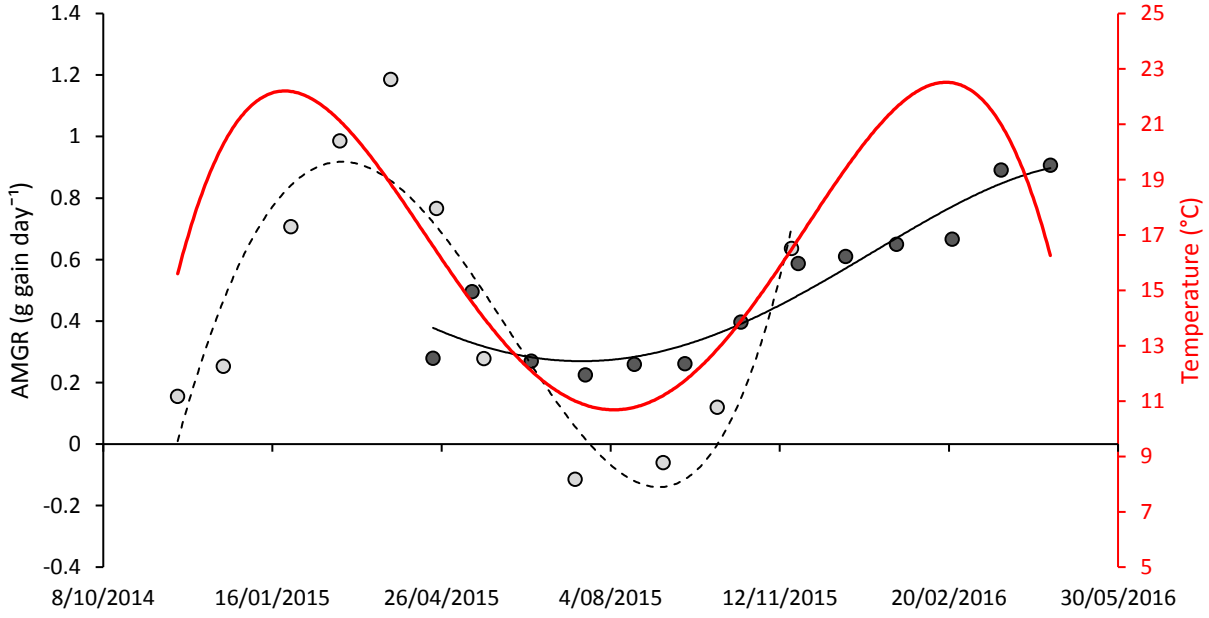

Figure 2.28. Absolute mass growth rate (AMGR) of snapper (black dashed curve and light grey symbols) and YEM (black curve and dark grey symbols). The rest of the Figure caption is the same as for Fig. 2.11.

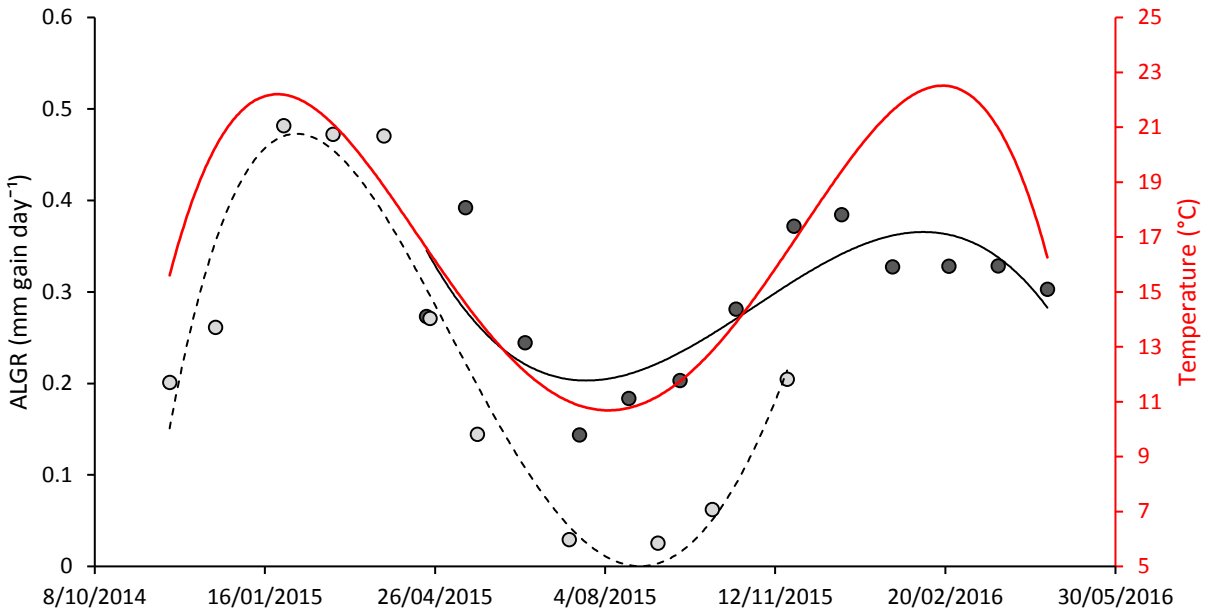

Figure 2.29. Absolute length growth rate (ALGR) of snapper (black dashed curve and light grey symbols) and YEM (black curve and dark grey symbols). The rest of the Figure caption is the same as for Fig. 2.11.

2.3.4.3.2 AMGR and ALGR

Absolute growth rates were more similar between the two species than specific rates in terms of correlation strength with the sea-water temperature (Fig. 2.28, 2.29). That was because YEM AGRs, especially ALGR (Fig. 2.28), more tightly followed the temperature curve than specific rates, and snapper continued showing the strong association with temperature (Fig. 2.28, 2.29). However, in terms of amplitude, the differences between species were even more emphasised for AGRs. Snapper exhibited ~90% and 85% higher differences between the lowest and highest data points than YEM for AMGR and ALGR respectively.

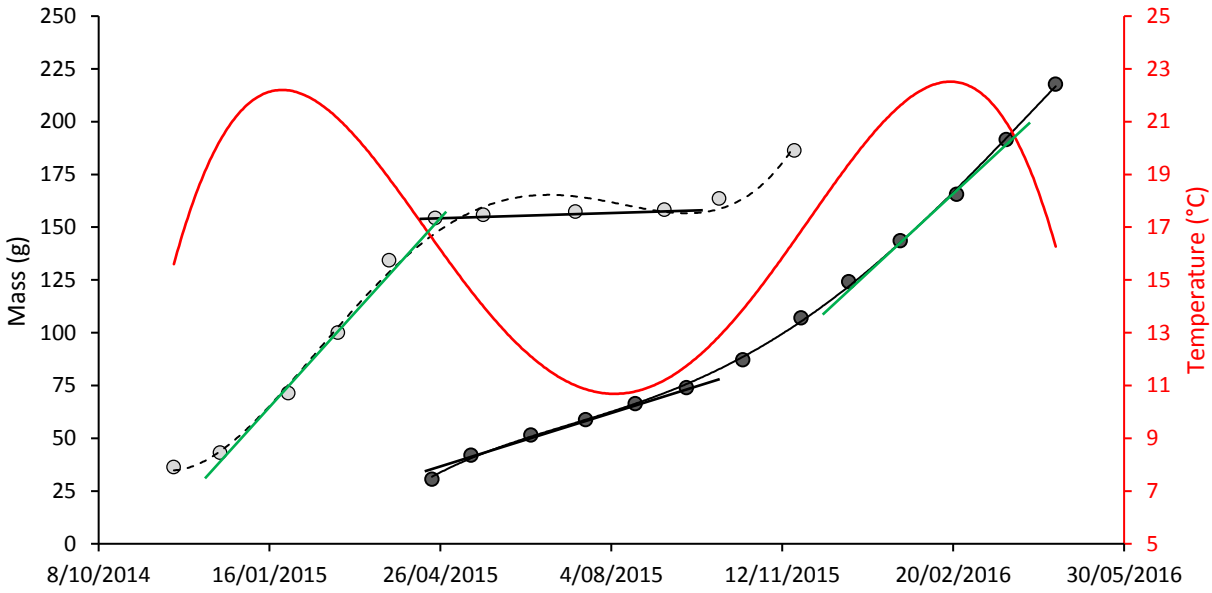

Figure 2.30. Absolute Cumulative Mass Gain (ACMG) of snapper (black dashed curve and light grey symbols) and YEM (black curve and dark grey symbols). Black and green lines associated with the ACMG data represent linear regression models explaining data for the period from April and September 2015 and December 2014 to March 2015 for snapper and YEM respectively (for explanation see text). The rest of the Figure caption is the same as for Fig. 2.11.

2.3.4.3.3 ACMG and ACLG

Absolute Cumulative Mass Gain between the two species showed a major deviation in their trajectories during winter time when snapper ceased to grow and YEM continued (Fig. 2.30). To statistically assess this observation, comparison of linear regression models belonging to the period from April to September 2015 for both species ACGs revealed significant differences (black lines Fig. 2.30, $F_{1, 6} = 37.12$, $p < 0.001$ and Fig. 30, $F_{1, 6} = 37.25$, $p < 0.001$ for AMGR and ALGR respectively). Another, less obvious difference can be observed with closer statistical investigation of slopes representing summer growth of snapper and YEM (green lines Fig. 2.30, 2.31). When summer time data (December 2014 to March 2015) for ACMG was statistically analysed the level of significance was not strong enough for clear interpretation of the trend ($p = 0.069$, $F_{1, 4} = 6.07$). However, it became evident that snapper summer growth was superior to YEM when the same period of time from ACLG data were compared ($p < 0.001$, $F_{1, 4} = 302.84$, Fig. 2.31).

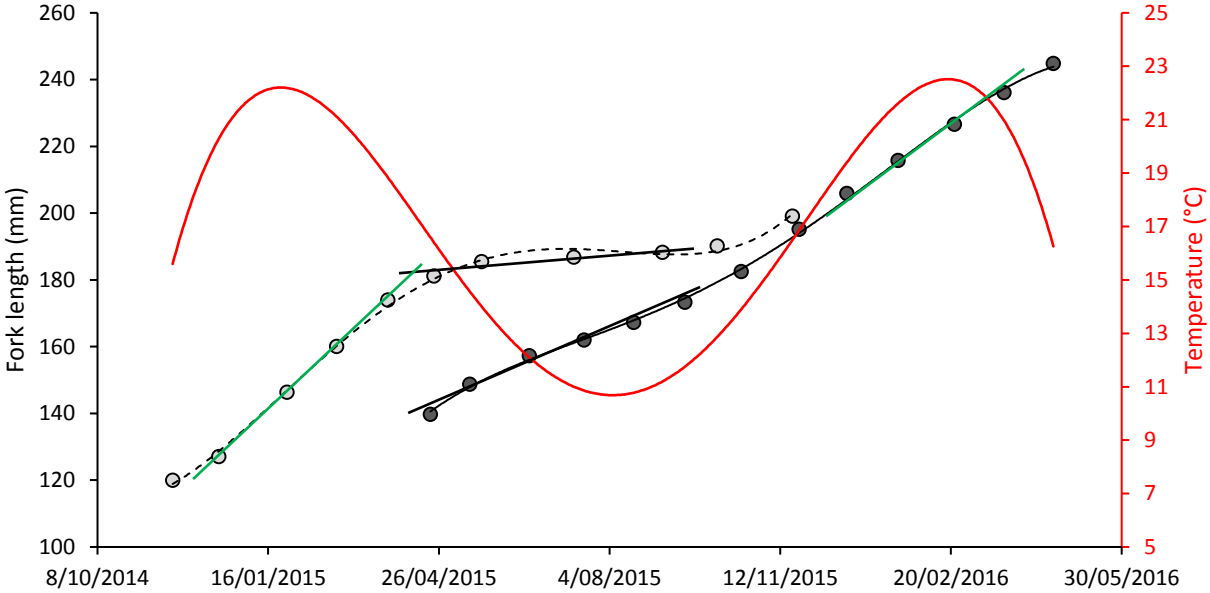

Figure 2.31. Absolute Cumulative Length Gain (ACLG) of snapper (black dashed curve and light grey symbols) and YEM (black curve and dark grey symbols). The rest of the Figure caption is the same as for Fig. 2.30.

2.3.4.4 Daily Feed Consumption (DFC) – percentage of feed consumed by estimated total fish mass tank^{-1} day^{-1}

Snapper DFC was highly correlated with sea-water temperature (Fig. 2.32; Table 2.4), bringing further evidence to support the view that temperature is one of the major drivers of snapper growth.

In the case of YEM, as depicted in Fig. 2.33, temperature appeared only partially related with daily feed intake throughout the experiment, which resulted in a non-significant correlation between the two datasets (Table 2.4).

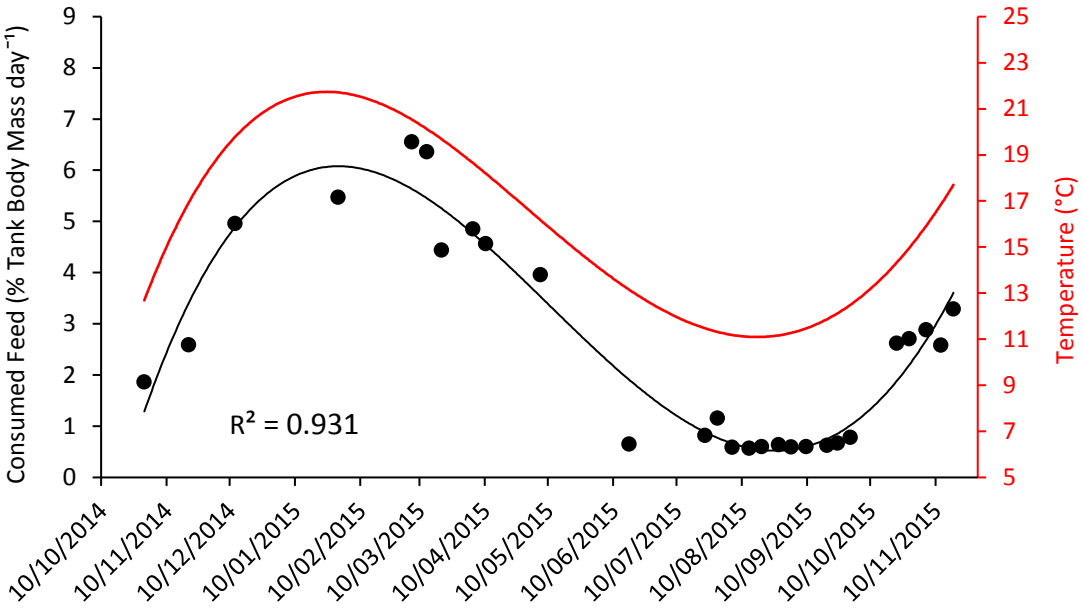

Figure 2.32. Daily Feed Consumption (DFC), percent of feed consumed per day per tank (black curve and black symbols) based on estimated tank body mass of snapper.

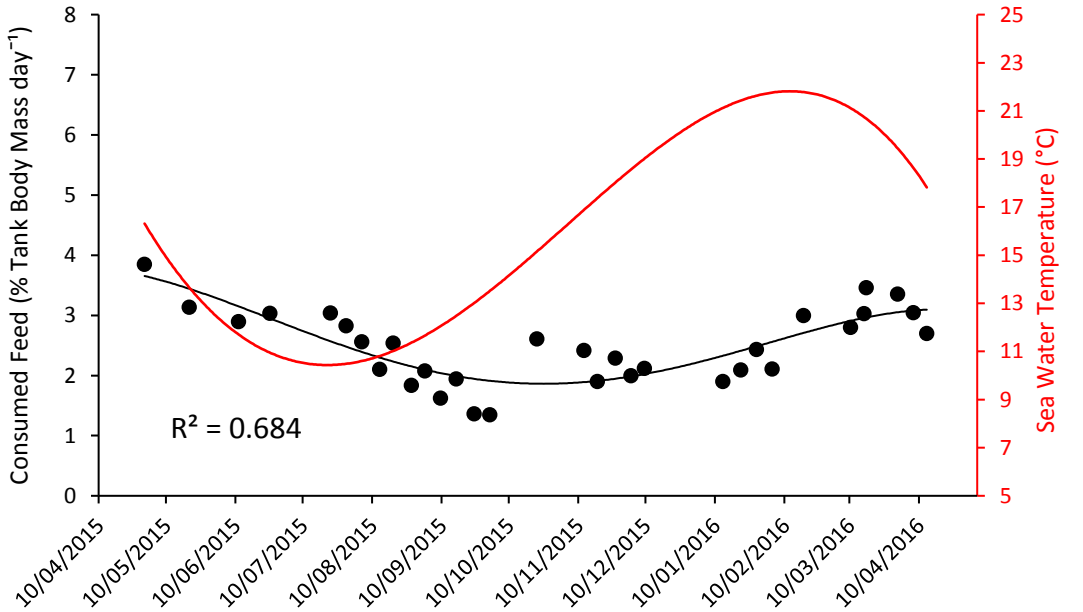

Figure 2.33. Daily Feed Consumption (DFC) percent of feed consumed per day per tank (black curve and black symbols) based on estimated tank body mass of YEM.

2.3.5 Organosomatic indices and supplementary morphometrics

2.3.5.1 Snapper and YEM Body indices – effect of temperature

2.3.5.1.1 Snapper

In this section the importance of utilising the curve smoothening function based on 4th polynomial order to predict data based on the generated curve revealed certain trends that would otherwise remain overlooked.

Both sets of data (predicted and raw) of the gutted mass index (GMI) demonstrated moderately strong negative correlation when regressed against sea-water temperature (Table 2.5, Fig. 2.34A). For cardio-somatic index (CSI), however, only with predicted data a possible trend based on a moderately strong negative correlation with sea-water temperature surfaced (Table 2.5, Fig. 2.34B). When the first data pair (i.e. November 2014) was omitted the trend became obvious (predicted data, $R = 0.693$, $R^2 = 0.481$, $F_{1, 9} = 7.40$, $p = 0.026$). In the case of the spleno-somatic index (SSI) no significant correlation was detected (Table 2.5, Fig. 2.34C). The hepato-somatic index (HSI) on the other hand with both raw and predicted datasets supported the graphic suggestion of a strong negative correlation with the sea-water temperature (Fig. 2.34D, Table 2.5). The entero-somatic index (ESI) was another cryptic dataset where no association with sea-water temperature was evident on raw data alone. Even though 4th polynomial function on raw ESI data did not produce a great fit ($R^2 = 0.37$), when those predicted values were regressed against the temperature a strong positive correlation was detected (Table 2.5, Fig. 2.34E). The visceral-lipid index (VLI) data, were moved three data points (months) forward on the temperature scale in order to test correlation with temperature, as suggested by Fig. 2.34F, where a lag-time was illustrated. This resulted in detecting a strong correlation with sea-water temperature (Table 2.5).

2.3.5.1.2 YEM

GMI exhibited a decreasing tendency for approximately the first 6 months in the study. For the rest of the experimental period GMI was maintained at that level with slight oscillations observed between February and April 2016 (Fig. 2.35A). This trajectory did not produce a significant correlation with temperature, whereas HSI predicted values (i.e. raw data were not significant) with seawater temperature showed a moderate, negative correlation (Table 2.5, Fig. 2.35B). SSI had peculiar annual dynamics with the appearance of a positive correlation with sea-water temperature for the first 7–8

months of the study and negative correlation for the rest (Fig. 2.35C). However, there was no significant correlation found when entire SSI data were tested against temperature. HSI, generally, demonstrated the same yearly dynamics as snapper with a detection of a strong negative correlation with sea-water temperature (Table 2.5, Fig. 2.35D). ESI behaved in the same fashion as GMI, tending to gradually decrease until the beginning of summer when a drop was stabilised for the rest of the study (Fig. 2.35E). Consequently, there were no observed significant correlation with temperature. Graphic expression, as well as statistical analysis of VLI (Fig. 2.35F) did not reveal an association with temperature.

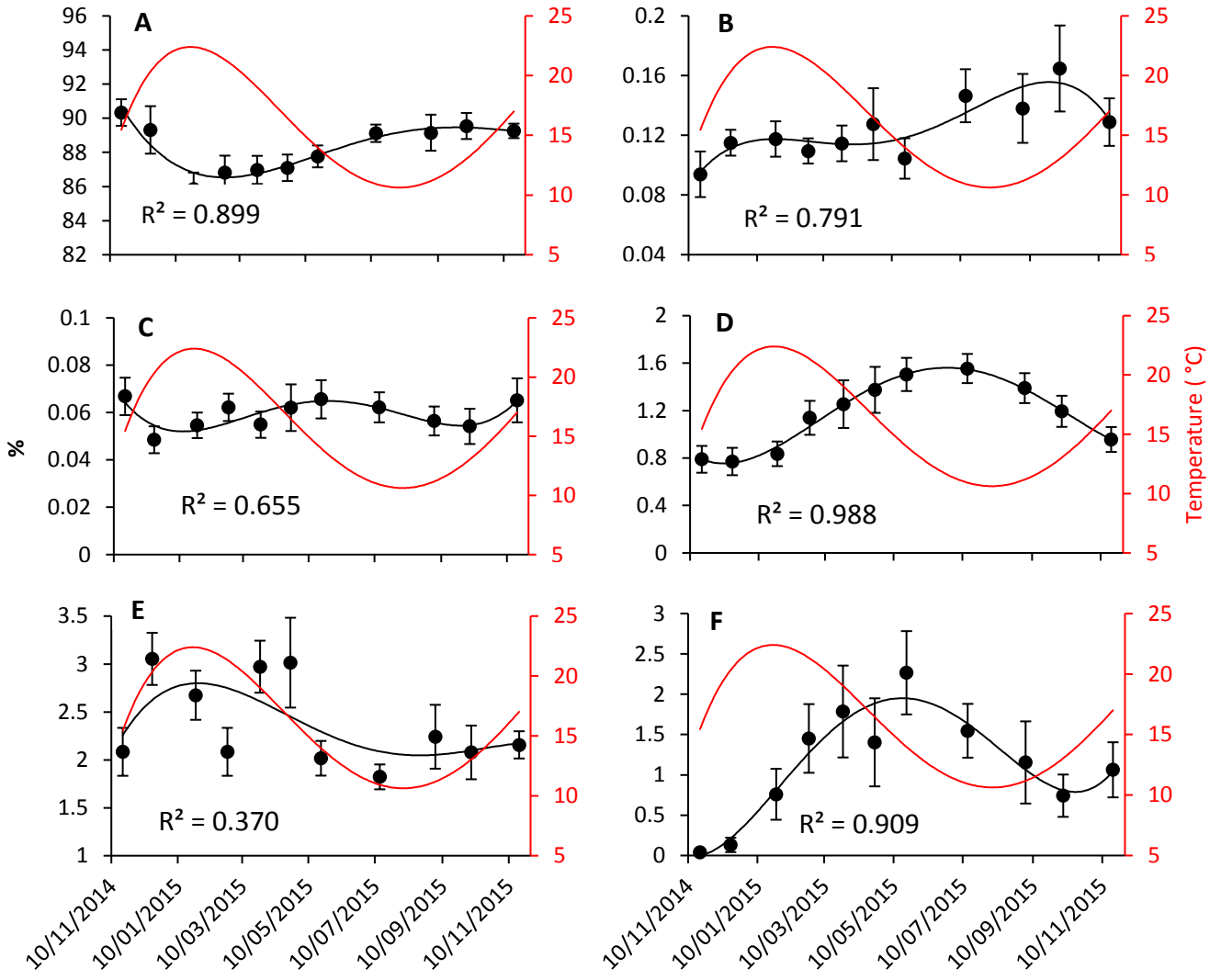

Figure 2.34. Organosomatic indices and supplementary morphometrics of snapper (black curve, and black symbols) superimposed with sea-water temperature curve (red). A – Gutted mass index (GMI), B – Cardio-somatic index (CSI), C – Spleno-somatic index (SSI), D – Hepato-somatic index (HSI), E – Entero-somatic index (ESI), F – Visceral-lipid index (VLI).

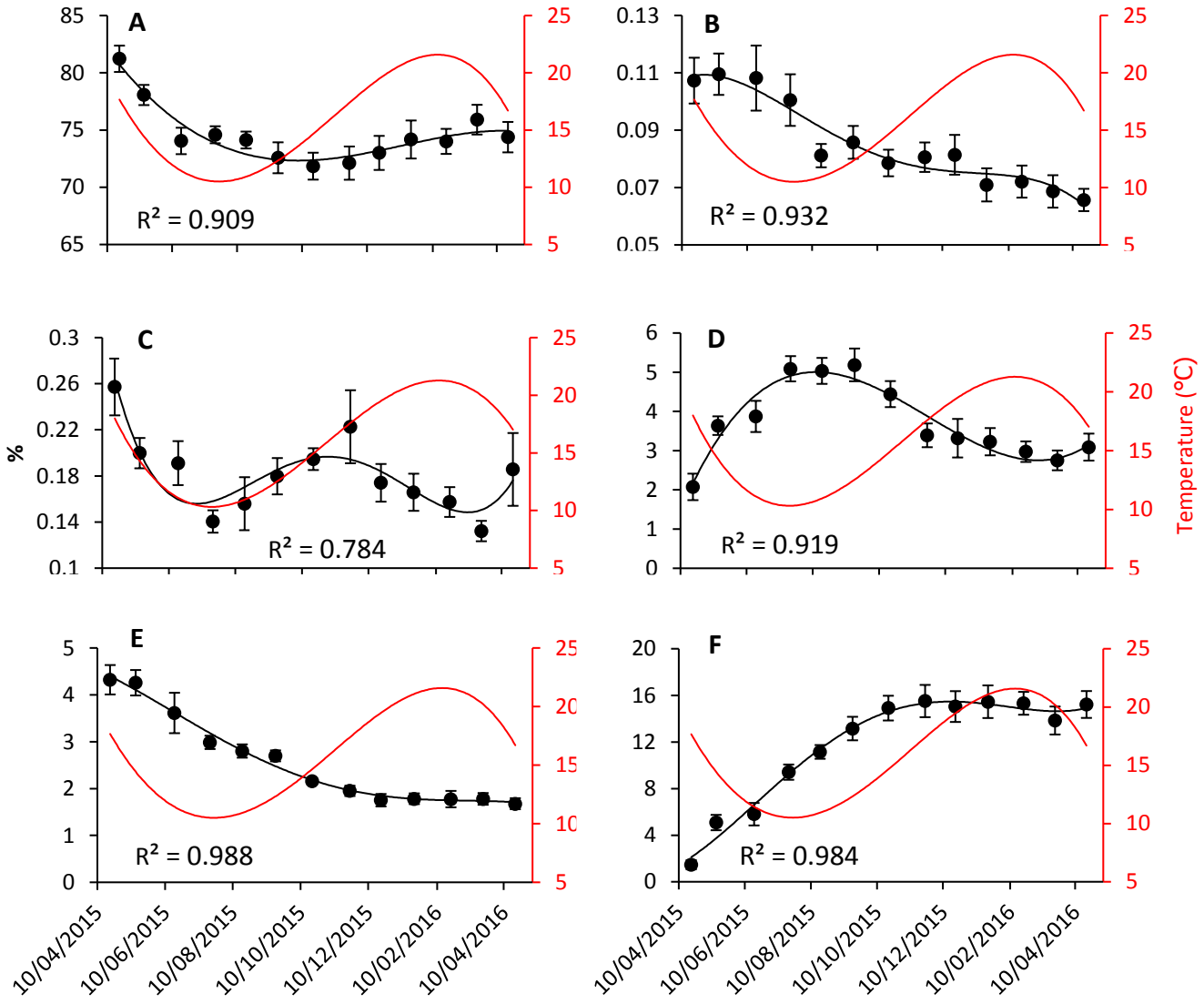

Figure 2.35. Organosomatic indices and supplementary morphometrics of YEM. The rest of the Figure caption is the same as for Fig. 2.34.

2.3.6 Visceral organs and tissue correlations with body mass

Dynamics of snapper and YEM internal organs/visceral fat deposits/gutted body mass including fork length, through the period of 12 months, can be also illustrated by examining how well they correlate with the total body mass (Fig. 2.36 and 2.37 for snapper and YEM respectively). In the case of snapper, when the dynamics is superimposed with the temperature curve it becomes apparent that

certain periods during the calendar year (i.e. early spring and late autumn) that correspond with sea-water temperature ranging from 16 to 20°C, exhibit the highest and more uniform correlations than any other periods. When the same YEM data was presented with the temperature curve, the highest and the most steadfast correlations occurred from mid-winter till late spring, again in contrast to snapper, however, matching with the period when lipid deposition increased linearly until maximum levels were reached coinciding with times of the termination of the uniform correlation period (Fig. 2.37).

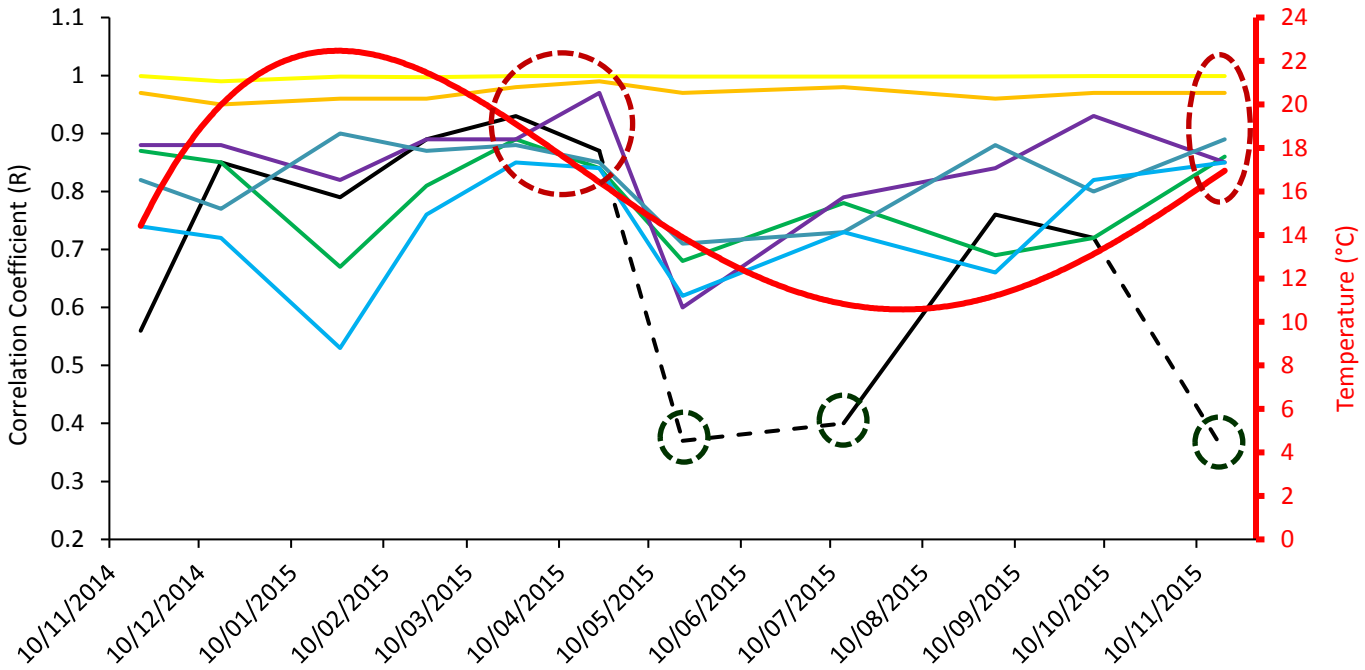

Figure 2.36. Internal organs (heart — , intestines with pyloric caeca — , liver — and spleen —) mass, visceral fat deposits — mass, gutted body — mass, and fork — length of snapper correlations with total body mass through period of the 12-month growth study superimposed with temperature curve (red). Temperature curve was derived from raw data fitted to 4th polynomial function. A red dotted circle and ellipse show when correlations are grouped together at their highest level. Dark green dashed circles and associated dashed black lines represent non-significant correlations of heart with the total body mass in May, July and November 2015.

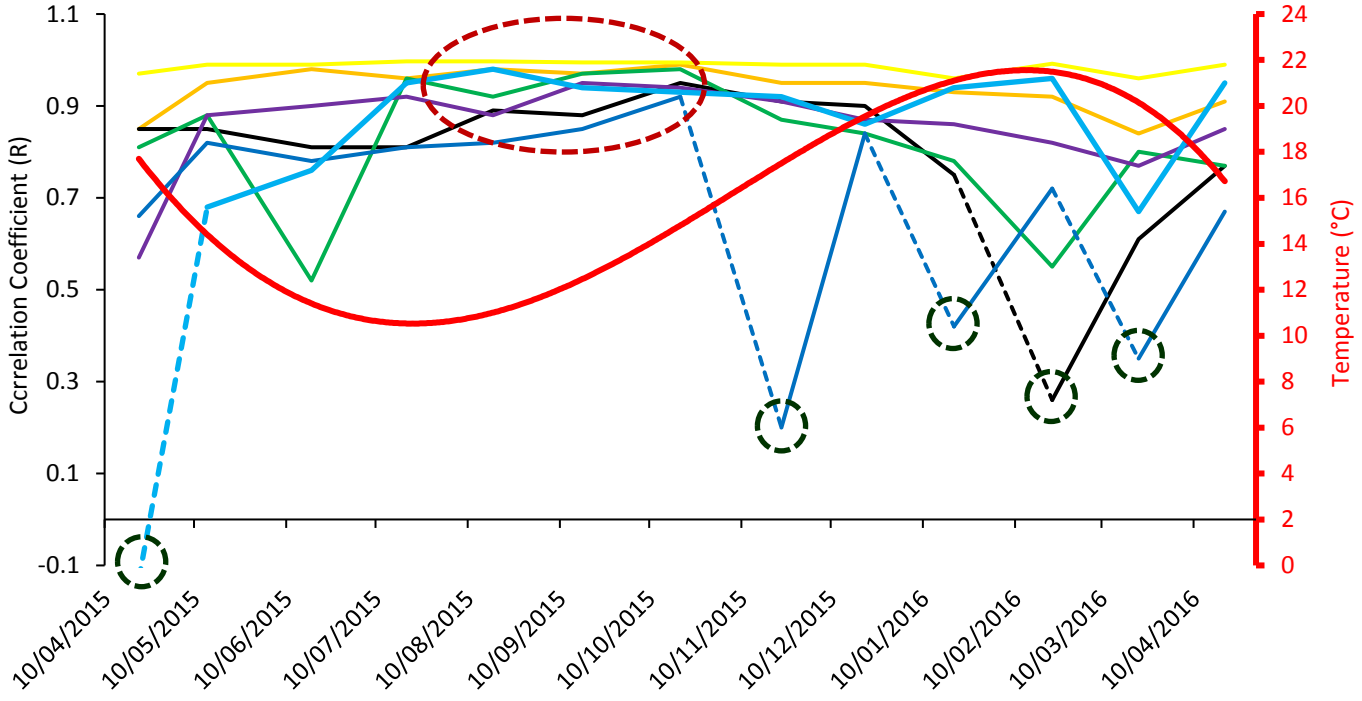

Figure 2.37. Internal organs (heart ─ , intestines with pyloric caeca ─ , liver ─ and spleen ─) mass, visceral fat deposits ─ mass, gutted body ─ mass and fork ─ length of YEM correlations with total body mass through period of the 12-month growth study superimposed with temperature curve (red). A red dashed ellipse show when correlations are grouped together at their highest level. Dark green dotted circles and associated dashed light blue (visceral fat; April 2015), dark blue (spleen; November 2015, January and March 2016) and black (heart; February 2016) lines represent non-significant correlations.

Table 2.4. Growth parameter correlations with sea-water temperature for the duration of the growth study for snapper and YEM calculated with raw or raw and 4th polynomial predicted data as described in 2.2.6.1, with associated 4th polynomial R^2, correlation coefficient with its R^2, F-test statistics, p-values, lag-time value (if applicable, number of parameter data points [months] move on the temperature scale) and correlation direction. Bold and italic represent the data with significant and non-significant correlation with temperature respectively.

Species		Data Type	4th polynomial R^2	Correlation Coefficient	R^2	F Statistics	p-values	Lag-time	Direction
SN	**Rm**	**RAW**		**0.88**	**0.79**	**31.71**	**< 0.001**	**3**	**POSITIVE**
		PREDICTED	0.95	n/a	n/a	n/a	n/a	n/a	n/a

	SMGR	**RAW**		**0.96**	**0.92**	**108.66**	**< 0.001**	**n/a**	**POSITIVE**
		PREDICTED	0.92	n/a	n/a	n/a	n/a	n/a	n/a
	AMGR	**RAW**		**0.81**	**0.65**	**16.87**	**0.003**	**n/a**	**POSITIVE**
		PREDICTED	**0.84**	**0.81**	**0.66**	**17.61**	**0.002**	**n/a**	**POSITIVE**
	ACMG	**RAW**		**0.66**	**0.44**	**6.97**	**0.027**	**1**	**NEGATIVE**
		PREDICTED	0.99	n/a	n/a	n/a	n/a	n/a	n/a
	SLGR	**RAW**		**0.96**	**0.91**	**96.29**	**< 0.001**	**n/a**	**POSITIVE**
		PREDICTED	0.94	n/a	n/a	n/a	n/a	n/a	n/a
	ALGR	**RAW**		**0.95**	**0.9**	**79.39**	**< 0.001**	**n/a**	**POSITIVE**
		PREDICTED	0.93	n/a	n/a	n/a	n/a	n/a	n/a
	ACLG	**RAW**		**0.63**	**0.4**	**6.03**	**0.036**	**1**	**NEGATIVE**
		PREDICTED	0.99	n/a	n/a	n/a	n/a	n/a	n/a
	DFC	**RAW**		**0.96**	**0.92**	**302.69**	**< 0.001**	**n/a**	**POSITIVE**
		PREDICTED	0.93	n/a	n/a	n/a	n/a	n/a	n/a
YEM	**Rm**	**RAW**		**0.62**	**0.39**	**6.92**	**0.023**	**n/a**	**NEGATIVE**
		PREDICTED	0.98	n/a	n/a	n/a	n/a	n/a	n/a
	SMGR	*RAW*		*0.031*	*0.001*	*0.010*	*0.920*	*n/a*	*n/a*
		PREDICTED	*0.74*	*0.018*	*0.000*	*0.004*	*0.954*	*n/a*	*n/a*
	AMGR	**RAW**		**0.75**	**0.57**	**14.45**	**0.003**	**n/a**	**POSITIVE**
		PREDICTED	**0.9**	**0.79**	**0.63**	**18.72**	**0.001**	**n/a**	**POSITIVE**
	ACMG	**RAW**		**0.65**	**0.43**	**8.16**	**0.016**	**n/a**	**POSITIVE**
		PREDICTED	0.99	n/a	n/a	n/a	n/a	n/a	n/a
	SLGR	*RAW*		*0.31*	*0.09*	*1.14*	*0.309*	*n/a*	*POSITIVE*
		PREDICTED	*0.53*	*0.39*	*0.15*	*1.92*	*0.194*	*n/a*	*POSITIVE*
	ALGR	**RAW**		**0.75**	**0.56**	**14.11**	**0.003**	**n/a**	**POSITIVE**
		PREDICTED	**0.56**	**0.97**	**0.94**	**173.86**	**< 0.001**	**n/a**	**POSITIVE**
	ACLG	**RAW**		**0.67**	**0.45**	**9**	**0.012**	**n/a**	**POSITIVE**
		PREDICTED	0.99	n/a	n/a	n/a	n/a	n/a	n/a
	DFC	*RAW*		*0.15*	*0.02*	*0.71*	*0.408*	*n/a*	*POSITIVE*
		PREDICTED	*0.68*	*0.18*	*0.03*	*1.05*	*0.312*	*n/a*	*POSITIVE*

Legend: Rm – relative mass, SMGR – specific growth rate, AMGR – absolute mass growth rate, ACMG – annual cumulative mass gain, SLGR – specific length growth rate, ALGR – absolute length growth rate, ACLG – annual cumulative length gain, DFC – daily feed consumption.

Table 2.5. Body indices correlations with sea-water temperature for the duration of the growth study for snapper and YEM. The rest of the Table caption is the same as for Table 2.4.

Species		Data Type	4th polynomial R^2	Correlation Coefficient	R^2	F statistics	p-values	Lag-time	Direction
SN	**GSI**	**RAW**		**0.62**	**0.38**	**5.52**	**0.043**	**n/a**	**NEGATIVE**
		PREDICTED	**0.9**	**0.63**	**0.39**	**5.82**	**0.039**	**n/a**	**NEGATIVE**
	CSI	RAW		*0.53*	*0.28*	*3.51*	*0.093*	*n/a*	*NEGATIVE*
		PREDICTED	*0.79*	*0.58*	*0.34*	*4.59*	*0.061*	*n/a*	*NEGATIVE*
	SSI	RAW		*0.27*	*0.07*	*0.72*	*0.42*	*n/a*	*NEGATIVE*
		PREDICTED	*0.65*	*0.36*	*0.13*	*1.37*	*0.272*	*n/a*	*NEGATIVE*
	HSI	**RAW**		**0.64**	**0.41**	**6.18**	**0.035**	**n/a**	**NEGATIVE**
		PREDICTED	0.99	n/a	n/a	n/a	n/a	n/a	n/a
	ESI	RAW		*0.56*	*0.32*	*4.24*	*0.069*	*n/a*	*POSITIVE*
		PREDICTED	**0.37**	**0.92**	**0.85**	**51.22**	**< 0.001**	**n/a**	**POSITIVE**
	VLI	**RAW**		**0.78**	**0.61**	**14.04**	**0.005**	**3**	**POSITIVE**
		PREDICTED	0.91	n/a	n/a	n/a	n/a	n/a	n/a
YEM	GSI	RAW		*0.09*	*0.01*	*0.11*	*0.75*	*n/a*	*POSITIVE*
		PREDICTED	0.91	n/a	n/a	n/a	n/a	n/a	n/a
	CSI	RAW		*0.55*	*0.3*	*4.71*	*0.053*	*n/a*	*NEGATIVE*
		PREDICTED	**0.93**	**0.57**	**0.32**	**5.27**	**0.043**	**n/a**	**NEGATIVE**
	SSI	RAW		*0.01*	*0*	*0*	*0.98*	*n/a*	*n/a*
		PREDICTED	*0.78*	*0.01*	*0*	*0*	*0.98*	*n/a*	*n/a*
	HSI	**RAW**		**0.79**	**0.63**	**18.57**	**0.001**	**n/a**	**NEGATIVE**
		PREDICTED	0.92	n/a	n/a	n/a	n/a	n/a	n/a
	ESI	RAW		*0.52*	*0.27*	*4.06*	*0.069*	*n/a*	*NEGATIVE*
		PREDICTED	0.99	n/a	n/a	n/a	n/a	n/a	n/a
	VLI	RAW		*0.416*	*0.173*	*2.303*	*0.157*	*n/a*	*POSITIVE*
		PREDICTED	*0.98*	*0.417*	*0.173*	*2.309*	*0.157*	*n/a*	*POSITIVE*

Legend: GSI – gutted-somatic index, CSI – cardio-somatic index, SSI – spleen-somatic index, HSI – Hepato-somatic index, ESI – entero-somatic index, VLI – visceral-lipid index.

2.4 Discussion

2.4.1 Growth observations

During 12 months under the satiation feeding regime, snapper demonstrated variations in growth rates that were markedly affected by seasonal oscillations in environmental conditions, as observed predominantly through strong positive correlations with sea-water temperature. Yellow-eyed mullet (YEM) showed a markedly different growth strategy with changes in specific growth rates (SGR) as well as absolute mass/length gain showing overall lack of seasonal and/or temperature effect. Temperature related changes in growth in snapper spanned from negative growth (i.e. weight loss) in winter to peaks in summer when water temperatures were the highest, whereas in YEM maximal SGR was observed between 17 and 18°C. In much the same pattern, snapper feed consumption was related to annual growth rates and temperature, therefore snapper feeding behaviour and the resulting feed intake appeared to be temperature dependent. YEM feed intake was aligned with SGR but not with temperature, which demonstrated that YEM possess a growth strategy with the capacity to minimise temperature effects on growth performance.

The typical transitional or stepwise annual growth pattern observed for snapper by earlier research (e.g. Francis, 1994a) and this study mimics the ontogenetic growth pattern of the species where soon after metamorphosis (in summer) growth rate accelerates substantially (Smith and Hataya 1982; Foscarini, 1988; Francis, 1994a; Sim-Smith et al., 2012), but during their first winter, growth rates are suppressed (Lenanton, 1974; Paul, 1976; Bell et al., 1991). This growth pattern repeats until maturation, when growth intensity slows down (Francis, 1994a). The annual/seasonal growth trend exhibited by snapper in this and other studies compares to that commonly observed for other temperate sparids, such as red seabream, *Pagrus major* (Foscarini, 1988) and gilthead seabream, *Sparus aurata* (Perez-Sanchez, 1994; Kissil et al., 2001; Ibarz et al., 2003, 2010). *P. major* found around Japan may experience a similar annual temperature range (i.e. 10–23°C) as New Zealand snapper, and a frequently reported threshold below which seabream suspend consuming food and growth ceases (i.e. 10–12°C; Foscarini, 1988; Kim, 2000) was also found to be a match with *C. auratus*. Snapper's Mediterranean sister species gilthead seabream (e.g. Kissil et al., 2001) demonstrated an annual growth pattern where the second winter did not suppress growth to the level of cessation, which was possibly due to overall higher annual temperatures. When approaching winter, *S. aurata* reduce food intake congruently with the drop in sea-water temperature and when the threshold (12–13°C) is reached food consumption is markedly reduced causing a significant growth reduction. This pattern

of food intake and growth is mimicked by snapper in this study, where growth and feed intake decreased markedly below 11.5°C.

In winter, cultured *S. aurata* are subject to the onset of a condition termed "winter syndrome" (Ibarz et al., 2003, 2010). Sparids and particularly *S. aurata* seem to be sensitive to low temperature and this trait in wild populations seems to be related to a winter movement to deeper, warmer and more temperature stable waters (Davis, 1988). Cultured fish, especially from the northern Mediterranean (42–45 °N) are forced to face lower winter temperatures and often develop the winter syndrome condition which is characterised by overall increased mortality rate, lethargic and erratic behaviour (e.g. separation and swimming on one side), expression of typical skin pigmentation stress pattern (i.e. darker skin and vertical bands) and skin lesions (Contessi et al.,2000; Ibarz et al., 2010). The affected fish are normally in the category of 100–400 g and they appear to be in relatively good condition (Ibarz et al., 2010). In the present study, snapper showed several of these symptoms, especially the appearance of skin lesions with approximately 30 % of fish affected.

YEM growth, specifically with an emphasis on seasonal effects, has not received much attention in the primary literature. Nevertheless, Chub at al. (1981) reported annual growth patterns of YEM from the Swan-Avon River System in Western Australia where winter growth was still present, though somewhat reduced in comparison with other seasons, and the winter growth rates observed in the present study. This may suggest the importance of food limitation for YEM growth in the wild (for details see Chapter 6) and that YEM has a capacity to grow even during winter providing adequate food is supplied. The strength of the latter suggestion could be further supported by the fact that flathead grey mullet, *Mugil cephalus*, which were simultaneously monitored in Chub et al., (1981), exhibited typical temperate winter growth suppression, while YEM continued growing. The observations of notable winter growth in this study are not generally observed in other mugilids including striped mullet, (*Mugil platanus*; Castro et al., 2009) and leaping grey mullet (*Liza saliens*, Cardona, 1999a, b). Examples of winter growth, however, at three to four times reduced rates compared with summer, have been reported for juvenile *M. cephalus,* for example, in Anderson (1958) for fish from the Atlantic coast of USA, and in Rossberg and Strawn (1980) for pond reared fish in polyculture. Reports describing mullet growth performance usually come from the Northern Hemisphere, where mullet seem to experience larger annual temperature fluctuations (i.e. 10°C in winter to 30°C in summer), especially for species from the Mediterranean Sea (e.g. Cardona 1999a; 1999b), than YEM in New Zealand, which may contribute to the pronounced seasonal growth differences compared to the findings associated with this study. Therefore, it appears that, according to the author's knowledge, YEM is the only mugilid with a unique annual growth dynamic characterised with a potential for proper winter growth.

It has been considered by Guderley et al. (1996) that an inconsistency in food availability, particularly in temperate regions, has a strong seasonal component. This reduction in feeding resources are primarily associated with the winter season, and fish generally respond to this prolonged decrease in food supply by reducing metabolic expenditures and altering tissue metabolic capacities (Pelletier et al., 1993; Yang and Somero, 1993). Such a notion has been reported in many other instances (e.g. Bjornosson, 2001; Metcalfe et al., 2002; Bacon et al., 2005) whereby in addition to reduced temperatures, food availability may play a substantial role in the growth depression. In the present study the Daily Feed Consumption (DFC, percentage of feed consumed relative to estimated total fish mass day^{-1}) of snapper showed a strong correlation with annual sea-water temperature. However, this cannot be said for YEM where no clear annual/seasonal pattern in DFC was observed, further highlighting differences in the behaviour and physiology of these two species. Moreover, YEM consumed on average less relative amount of food day^{-1} than snapper, which was approximately maintained equal throughout the entire experiment.

Further evidence of these contrasting feeding behaviours between snapper and YEM can be found in Coubrough et al. (2004), who were exploring seasonal changes in feeding pattern between the two species. In their annual demand feeding experiment snapper hardly fed below 11°C, whereas feeding between 11 and 18°C varied 100 to 500 feeder activations day^{-1} and was distinctly increased above 18°C reaching on average over 1500 activation day^{-1}. Minimum feeding activity was in August (austral winter) and maximum in January (austral summer) showing seasonal effects, with step-wise feeding stages rather than a linear relationship (i.e. gradual increase) between feeding levels and temperature. This was consistent with the present study where specific growth rates were the lowest below 11°C and the highest above 18°C. YEM, however, as reported in Coubrough et al. (2004), exhibited slightly oscillating but generally consistent annual feeding behaviour resembling the present findings. Similar patterns to YEM's have been observed for pike, *Esox lucius* (Johnson, 1966), and yellowtail, *Seriola quinqueradiata* but only for temperatures above 18°C (Kohbara et al. 2003). Interestingly, below 18°C, yellowtail demonstrated the same trend in feeding behaviour with transitional temperature levels of 12 and 17°C, similar to snapper. One of the suggestions from Coubrough et al. (2004) for relatively consistent YEM feeding rate was that mullet are eurythermal, displaying a low thermal threshold below 11°C. Nevertheless, this was not supported with the current results, indicating the possibility that the low thermal threshold for YEM may be below 11°C. However, YEM white muscle was found to have significantly higher concentrations of inorganic phosphate, creatine and mitochondria in winter compared with summer acclimated fish indicating the occurrence of a seasonal adjustment in metabolism (Black, 2002).

With reference to the present study the concept that a winter decrease in food supply accounts for a drop in growth rates cannot be supported with respect to snapper, where food supply was

unrestricted throughout the year and yet the winter growth reduction pattern was still evident. A similar argument was also found in other studies (e.g. Karaås 1990; Griffiths and Kirkwood, 1995). Therefore, winter feed intake cannot be explained by the food availability alone since feed intake and satiation have been found to be regulated possibly through interactions between physiological, social and environmental factors (Tran-Duy et al., 2008). However, for the YEM, growth can be maintained throughout the year when food supply is unrestricted. Thus, if any seasonal reduction in growth were to be observed it is plausible that the lack of food could explain this observation.

2.4.2 Resource partitioning observations

To maximise survival, fish have to balance between two fundamental ecological aspects – accelerating somatic growth when conditions are favourable, and replenishing energy stores by altering direction of resource partitioning to increase their chances to withstand the cold season (Post and Parkinson, 2001; Dıaz et al., 2009). For temperate juvenile fish this principle is most notable in summer where it is often observed that the majority of available resources are used to optimise growth (Hurst and Conover, 2003; Biro et al., 2005; Huss et al., 2008). However, in autumn energy utilisation is redirected towards maximising energy reserve stores (Hurst and Conover, 2003; Biro et al., 2005; Huss et al., 2008). Much like wild post larval snapper (0+, Sim-Smith, 2013a), the juvenile snapper (1+) utilised in the present study intensified visceral fat deposition towards the end of summer indicating that resource allocation switched from maximising growth in mid-summer to maximising energy storage until late autumn/early winter when this switch was at its peak. Subsequently visceral fat deposits were utilised and progressively depleted as winter advanced. Therefore, it seems that maximising chances for overwintering survival is potentially of higher ecological priority than to get bigger and thus less prone to predation at this age of their development. Or, in other words, unfavourable winter conditions may be a higher threat to juvenile snapper survival than potential predation (Sim-Smith, 2013a; present study).

The stimulus for the metabolic switch from growth to energy storage was often thought to be a low temperature threshold; however, Sogard and Spencer (2004) demonstrated that other variables such as fish size may also play an important role. They found that small immature sablefish, *Anoplopoma fimbria*, devoted more energy towards growth than larger fish regardless of water temperature, while fish acclimated to a colder environment did not necessarily generate larger energy stores compared with fish acclimated to higher ambient temperatures. Nevertheless, since small fish in the Sim-Smith et al. (2013a) study were partitioning resources irrespective of their length it has been suggested that the turning point for the switch from growth to stores was time and/or temperature dependent. In addition, the relationship between growth and energy stores can also be influenced by food

availability. Fish fed to satiation may maximise both aspects and a positive correlation is often found to describe growth and energy stores relationship, but limited (patchy) food supply generates a trade-off between the two variables with weak or lack of correlation (Sogard and Spencer, 2004; Jacobs et al., 2012). Results from the current study are only partially consistent with this observation since unrestricted food supply did produce a positive correlation between specific growth rates and relative visceral fat mass (i.e. visceral-lipid index, VLI). However, with approximately 2 months lag time this more resembles the dynamics described in Sim-Smith et al. (2013a).

Changes in animal condition, which reflect changes in growth and metabolic stores, have also been described in other studies. For example, Francis (1997) and Majed et al. (2002a) examined the annual patterns of wild snapper condition on a monthly/bimonthly basis. Both studies were located in the north-western Hauraki Gulf of North Island (New Zealand) and their findings indicated seasonal effects on juvenile snapper growth and condition. However, this was not evident from a monitoring of condition factor (i.e. Le Cren, 1951, relative condition factor, *Krel*) but rather by measuring annual trajectories of hepato-somatic index (HSI, Francis, 1997), or total RNA and protein indices (Majed et al., 2002a).

The present study, which characterised individual (rather than population) changes in condition in combination with a satiation feeding regime allowed for a clear identification of seasonal effects. Snapper condition expressed as relative mass in relation to mean mass (*Rm*, Froese 2006) had an explicit seasonal character since its annual trajectory followed the temperature oscillation curve (Fig. 6) with approximately 2–3 months' lag time. *Rm* data was also strongly positively correlated with VLI. Therefore, seasonal effects on juvenile snapper condition were detected in all three studies, further supporting the importance of environmental factors on snapper development and growth. However, annual condition pattern as portrayed using relative condition factor (*Krel*) in Francis (1997) and Majed et al. (2002a) did not resemble the course of snapper condition as depicted with *Rm* in the present study. Why might this be? The current study was carried out by controlling for food supply via excess feeding. This bypassed challenges that may arise in wild data where physiological or morphometric patterns can be confounded by the lack of regularity in the food supply or temporal and spatial food patchiness (Arnason et al., 2009). Francis (1997) and Majed et al. (2002a) utilised wild fish in their research and this may explain the reason why the indication of an annual cycle in *Krel* of juvenile and immature snapper was not clear. Furthermore, differences in growth patterns amongst New Zealand sites are commonly detected (Francis, 1994a, 1997), whereby snapper originating from Tasman/Golden Bay (north of the South Island, this study) have been shown to be faster growing than snapper from Hauraki Gulf (middle of North Island; Francis, 1997 and Majed et al., 2002a studies). And finally, temperature differences between the two locations may also be a contributing factor, since at least in Majed et al. (2002a) (in Francis, 1997, annual sea-water

temperature range has not been reported) lowest winter temperature in the Hauraki Gulf did not drop below 13°C, while in the present study snapper were exposed to temperatures below 10°C during winter. Like changes in condition factors, hepato-somatic index (HSI) can also identify changes in animal condition, growth, and nutritional state (Jensen, 1979). In addition to *Krel*, HSI, as mentioned earlier, was also a measure used in Francis (1997), which turned out to be a suitable metric to identify seasonal effects on juvenile snapper condition. It peaked in autumn/winter and dropped to the lowest level in summer mirroring the annual HSI pattern in the present study.

Since liver energy store utilisation during winter sounds more plausible than its replenishment (Grigorakis et al., 2002), the seasonal HSI pattern observed in Francis (1997) and in the present study appears to have a different course. Nevertheless, the observed pattern indicates that depletion of energy reserves during intense growth (all available resources are utilised for body growth, including those stored in liver) and their rebuild/restoration during the period of sluggish growth is an adaptive snapper trait (Francis 1997). In addition, Francis (1997) observed that the spring-summer period was characterised with high abundance of snapper in the surveyed area compared with autumn-winter when the abundance was low and he suggested that this density-dependent response to seasonal variations may be responsible for the intraspecific competition as an alternative explanation for the seasonal cycle in HSI. Nevertheless, the pattern where HSI was greater in winter relative to other seasons was also observed for other species, such as *S. aurata* (Grigorakis et al., 2002) and rainbow trout (*Salmo gairdneri*) (Hilton 1982). In addition, HSI measured in Francis (1997) had an inverse relationship with growth rate as was also observed in the present study. Therfore, in either study (present and Francis 1997) a significant correlation with growth rates was not observed, which may suggest that better nutritional state does not necessarily support faster growth (Francis, 1997).

In YEM an interesting pattern of feeding regulation and energy partitioning was observed. YEM annual condition dynamics, unlike in snapper, was negatively correlated with temperature, however VLI did not show an association with seasonal environmental changes but rather had a tendency to linearly increase until a plateau was reached in spring, 6 months after the start of the experimental feeding. The initial YEM response to unrestricted diet regime was manifested in a rapid increase in both condition and VLI, which coincided with the drop in sea-water temperature during autumn. This strong initial negative correlation with temperature that in turn significantly contributed to overall inverse correlation between *Rm* and sea-water temperature may be an artefact of adjustment to the *ad libitum* feeding regime rather than a consequence of decreasing temperature associated with autumn. YEM *Rm* was also aligned with relative liver mass in its annual trajectory. Therefore, similar to snapper, HSI can be also utilised as a predictor of seasonal variation in YEM condition. Although there has been virtually no work conducted on seasonal effects on YEM condition, a few publications on kin species demonstrated different seasonal dynamics to the one observed in this study. *M.*

cephalus from eastern Mediterranean, for example, steadily increased their condition until its peak in autumn which abruptly declined with approaching winter (Ozer et al., 2016); whereas condition factor of *Mugil curema* from South Brazil peaked in spring and was the lowest in winter without correlating with HSI (Albieri et al., 2010). However, *Mugil liza* from the same Brazilian region demonstrated the same positive association between condition and HSI as observed in the present study, but with the inverse seasonal trajectory to the one exhibited by YEM (i.e. *M. liza* condition factor and HSI were highest in summer and lowest in winter-autumn, Albieri and Araújo, 2010). The differences in condition and HSI between studies may not only be due to possible differences in growth capacity between species within the family Mugilidae but also because of differences in environmental conditions and their suitability for spawning, and larvae and juvenile fish development that may considerably vary amongst sites (Albieri et al., 2010). For instance, Western Australian YEM populations exhibit annual conditions that support summer spawning, while Eastern Australian and New Zealand populations have a winter spawning season (Chub at al., 1981). This coincided with the time in a year when the greatest condition and HSI were observed in the present study.

Based on positive correlations between SGR and DFC and inverse correlation between SGR and VLI observed in the present study, it could be argued that YEM sought to maximising accretion of fat stores (or devoting higher proportion of available energy to it) until an upper limit or optimal levels (i.e. ~15% BM) were reached. The ecological relevance for such a preference for retained lipid stores is not readily discernible, but may relate to a strategy that enables YEM to cope with patchy and temporally variable food supplies, or perhaps a strategy to improve gonadal fitness and reproductive potential. Therefore, YEM growth may be an indirect function of percentage of accumulated fat reserves, thus feed intake and growth rates may not be controlled by environmental cues as much as it is by the distance from ideal fat store levels since winter level of feed intake and fat stores deposition appeared to be independent of temperature (i.e. intake was relatively high, ~3%, and fat was accumulating in almost linear fashion throughout the coldest months).

2.4.3 Concluding remarks

Juvenile sparids, including *C. auratus*, are sensitive to temperature change in their environment since their growth rates and levels of feed intake are in close approximation with the annual temperature curve with the highest impact on their survival and wellbeing commonly observed below a 12°C threshold (Ibarz et al., 2010, 2003; this study). Snapper populations from Tasman/Golden Bay are adapted to the southern thermal niche of their geographical range, and consequently they may face the most severe overwintering conditions compared to conspecifics from other regions. Therefore, it appears that their growth strategy revolves around preparation (i.e. increasing condition

by switching resource partitioning from growth to energy stores) to increase odds of survival through the harsh winter period but with the maximum utilisation of the optimal conditions that expend over late spring – summer – early autumn, to amplify somatic growth crucial for supporting other ecological aspects such as overgrowing predators, sexual maturation and reproductive output. YEM utilised in the current study, on the other hand, seem to be comparably less affected by ambient temperature (i.e. only a mild distortion from annual linear growth was observed in winter, but the visceral fat deposition rate was not affected), possibly because their southern distribution frontier extends for a further 10° south (McDowall, 1978) and/or they possess different physiological growth capacity than sparids.

Therefore, annual alterations in growth rates are likely linked to temperature properties of the surrounding water that is known to affect diet, metabolism and controls the patterns of food intake (Person-Le Ruyet et al., 2004; Houlihan at al., 2008). Moreover, changes in growth are widely considered to be determined by the aerobic capacities of fish which are intrinsically associated with temperature (Jobling, 1994; Mallekh and Lagardère, 2002). In other words, growth is an explicit aerobic function where rate of oxygen utilisation is directly affected by temperature or by adaptation to a particular thermal niche, which is especially stressed for aquatic ectoderms (Fry, 1971; Jobling, 1994; Clarke and Fraser, 2004; Pörtner and Farrell, 2008). Therefore, an exploration of these relationships essential to maintain optimal fitness is paramount to understand underlying mechanism governing growth dynamics of juvenile temperate fish.

CHAPTER 3

Temperature effects on metabolic rates and aerobic scope of two coastal temperate species

3.1 Introduction

3.1.1 Metabolic rates – general overview and measurements

Two main processes – building new molecules and tissues (i.e. anabolism) and breaking down old tissues and ingested nutrients (i.e. catabolism) together with the biochemical processes engaged in body maintenance, locomotion and other activities are called metabolism (Jobling, 1994; Enberg et al., 2008). Metabolic activity can be measured as energy turnover in a unit of time, hence metabolic rate.

How is metabolic activity measured? Energy use in essential biochemical body reactions is thermodynamically inefficient and heat is released as a waste product (Nelson and Chabot, 2011). Fundamentally, energy turn-over, in simplified terms, revolves around the biochemical processes of creation and degradation of ATP (adenosine triphosphate) in the body (Nelson and Chabot, 2011; Nelson, 2016). Since there is no suitable apparatus to quantify ATP turnover (as possibly the best way to measure total metabolic activity), measuring the heat produced through the method of direct calorimetry became common practice (Jobling, 1994). However, for animals from an aquatic environment, direct calorimetry cannot produce valid results since the high heat capacity of water combined with the fish's relatively low metabolic activity generates a low signal to noise ratio (Jobling, 1994; Nelson, 2016). However, new technologies are emerging with the potential to overcome this challenge (Regan et al., 2013). Since ATP turn-over is strongly linked to utilisation of oxygen (O_2) and the simultaneous release of carbon dioxide (CO_2) (e.g. the complete aerobic respiration of a glucose molecule with standard vertebrate stoichiometry reads: $C_6H_{12}O_6 + 36ADP + 36P_i + 36H+ + 6O_2 \Rightarrow 6CO_2 + 36ATP + 42H_2O + Heat$), indirect calorimetry, which is based on measuring either the depletion of O_2 used-up by a fish from the surrounding water (i.e. oxygen consumption, MO_2) or a buildup of CO_2 produced by the fish, has been proposed as a method for measuring metabolic activity in aquatic organisms (Jobling, 1994). Since CO_2 in water is difficult to determine, measuring MO_2 has been accepted and used for determination of metabolic activity in aquatic animals for many decades (Cech, 1990;

Nelson and Chabot, 2011). However, using MO_2 as a surrogate for metabolic rates is not a faultless approach since two concerns, not accounting for the substrate being oxidized (i.e. composition of the diet) and contribution of anaerobic metabolism, outline possibilities of marked errors in expressing metabolic activity (Nelson and Chabot, 2011; Nelson, 2016). Regardless of these concerns MO_2 has become a standard measurement to express animal metabolism (Nelson and Chabot, 2011; Nelson, 2016). Nevertheless, the issue is less pronounced for fish since MO_2 was found to be a better approximation of metabolic activity of ectotherms than for endotherms, where the highest discrepancy between direct and indirect calorimetry was found (Walsberg and Hoffman, 2006). In addition, systematic preparation of experimental animals prior to measuring protocol should be carefully employed to minimise any mismatch between MO_2 and actual metabolic rates (Chabot et al., 2016).

3.1.2 Metabolic framework – resting versus maximum metabolic rate

3.1.2.1 Resting metabolic rates – overview

To satisfy comparability amongst animals (intra and interspecific), basal (BMR) or standard metabolic rates (SMR, 'standard' because it is specific for a given temperature) were established (Peters, 1986; McNab, 1997; Killen et al., 2007). BMR is the term commonly used where endotherms (e.g. mammals and birds) are concerned and it represents the minimum level of metabolic activity or energy expenditure, therefore it is an obligatory expense necessary to support life of individuals within the thermo-neutral zone when animals are at rest, post-absorptive (but not starving) and not in a growth or reproductive stage (Makarieva et al., 2008; Chabot et al., 2016). Even when an animal is at rest, not growing and post-absorptive, energy is required for renewal of macromolecules (e.g. proteins); for sustaining chemical homeostasis by moving ions or polar molecules across membranes or concentration gradients; and for mechanical work, such as the heart pumping blood, which is essential for preserving an organism's integrity (Nelson and Chabot, 2011). When ectotherms, fish in particular, are being measured the term SMR is used to represent resting rather than true basal metabolic rate. SMR is the closest measure to BMR possible and is obtained from animals under the same state as for BMR although at a temperature within their thermal tolerance zone that the given animal is acclimated to (Ikeda and Skjoldal, 1989; Seibel, 2007). Some fish (e.g. ambush or sit and wait predators) have distinct activity patterns between foraging and resting. Therefore, their resting rates can be measured and expressed as SMR (Nelson and Chabot, 2011). However, many other fish have in their behavioural repertoire different levels of spontaneous or compulsory activities (e.g. pelagic fish or continuously active obligate ram ventilators, like sharks), which makes measurement of SMR difficult. Therefore, routine metabolic rates (RMR) as resting rates are commonly measured (Dowd, 2006; Makarieva et al., 2008). The significance of knowledge of fish resting data is various. Maintenance metabolism has

intrinsic ecological relevance since it can determine fish behavioural traits (e.g. hierarchical dominance, social interactions and foraging) (Clarke and Johnston, 1999; Millidine et al., 2009; Killen et al., 2010; Metcalfe et al., 2016). Resting rates have been observed to increase with a shift to a high food ration and to decrease when a low food ration was introduced (Auer et al., 2015a). In the same study fish that increased resting metabolism more exhibited faster growth, but in response to a reduced meal size, individuals that lowered their maintenance metabolism most were better growers (Auer et al., 2015a). Resting metabolism is also an important component in energetic models that predict energy partitioning and resource allocation (Armstrong et al., 1992; Hansson et al., 1996), and finally it is an inevitable measurement for determination of aerobic scope (Fry, 1971).

3.1.2.2 Maximum metabolic rates (MMR) – overview

As SMR/RMR represents the lower, MMR indicates the upper boundary of aerobic capacity of an organism. When an animal is at the highest level of activity such that it is still supported by aerobic processes, MMR can be quantified (Fitzgibbon et al., 2007; Killen et al., 2007; Clark et al., 2013). This is commonly done by measuring MO_2 of fish during or just after exhaustive exercise, and two techniques are usually employed to elicit the highest aerobic metabolic expenditure. They are critical swimming tests in a swim-flume that is suitable for fish that are considered sustained swimmers (e.g. pelagic fish), and exhaustive chases, a technique appointed to stimulate MMR in fish that do not naturally exhibit prolonged swimming bouts (i.e. benthic species and ambush predators) (Reidy et al., 1995; Clark et al., 2013; Norin and Clark, 2016). MMR may vary considerably interspecifically due to differences in lifestyle, but intraspecific variations are also common and they are found to correlate with cardiac function, swimming performance and the ability to withstand environmental changes (Claireaux et al., 2005; Chatelier et al., 2006; Norin and Clark, 2016). Modulators often associated with change in MMR are body size (allometric effects), ambient temperature and oxygen availability, the three that are considered ecologically most relevant, but also osmoregulation, reproduction, digestion, and circadian and seasonal cycles may have significant effects on MMR (Wagner et al., 2005; Moran et al., 2014; Holt and Jørgensen, 2015; Norin et al., 2016).

3.1.2.2.1 Critical swimming speed (U_{crit})

Brett (1964) via his work on sockeye salmon, *Oncorhynchus nerka*, introduced a new protocol for simultaneous assessment of two important fish physiological variables, MMR and maximum sustained swimming capacity or critical swimming speed, U_{crit} (Kolok, 1999; Horodysky et al., 2011). Since then, U_{crit} has been used as a standard measurement to assess swimming capability of fish as an important

eco-physiological parameter (Plaut, 2001). After a fish is submitted to an exercise protocol in a swimming tunnel, water velocity is incrementally increased at prescribed intervals until the fish is not able to maintain its position in the water column and fatigue sets in (Plaut, 2001; Norin and Clark, 2016). The water velocity at the point of the fatigue represents the U_{crit} value, and it is thought to reflect maximum aerobic capacity or MMR at which point the cardiorespiratory system fails to further increase oxygen delivery to working tissues (Farrell, 2002; Steinhausen et al., 2008).

3.1.3 Temperature effects on aerobic scope

The two extreme metabolic values, resting metabolic rates (SMR/RMR) and MMR characterise boundaries between which any simultaneous aerobic activities above basal maintenance level, such as growth, digestion, locomotion and reproduction must be carried out and this is termed aerobic metabolic scope (Claireaux and Largardere, 1999; Behrens and Steffensen, 2007; Fitzgibbon et al., 2007; Killen et al., 2007; Clarke et al., 2013). When aerobic metabolic scope (AS) is determined by subtracting resting from MMR, absolute aerobic scope (AAS) is portrayed and when the scope is expressed as a ratio of maximum to resting metabolic rate, then it is referred to as the factorial aerobic scope (FAS) (Clarke, 2003; Clark et al., 2005; Clark et al., 2013; Farrell, 2016).

It has been proposed that to optimise fitness-related performance, such as growth, locomotion and reproduction, an organism should pursue a temperature range where AS is maintained at its highest levels (i.e. optimal temperature for aerobic scope, T_{optAS}) (Metcalfe et al., 2016; Nati et al., 2016). By defining T_{optAS} many researchers aimed to determine optimal conditions for fish growth and survival as an important ecological matrix especially in the light of global warming (Pörtner and Knust, 2007; Pörtner and Farrell, 2008; Pörtner, 2010). Within these considerations OCLTT or oxygen- and capacity-limited thermal tolerance hypothesis, which is based upon respiratory metabolism, sought to explain how temperature extremes constrain physiological performance in fish (Chabot et al., 2016; Farrell, 2016). The hypothesis proposes that the performance weakens as AS decreases when temperature rises above or drops below T_{optAS}, and therefore fish should strive to maintain their position within the range of T_{optAS} to optimise fitness and survival (Pörtner and Knust, 2007; Clark et al., 2013).

When ambient temperature approaches the upper boundary of the thermal tolerance, the capacity of physiological systems, mainly the cardio-respiratory system and aerobic capacity of tissues, eventually reach their threshold and become limiting (Farrell, 2002; Steinhausen et al., 2008; Pörtner, 2010). This state results in a distinct reduction in difference between resting and MMR, which impairs AS and further survival of the fish may be compromised (Steinhausen et al., 2008; Pörtner, 2010; Sandblom et al., 2014). The limitation may be derived from the combined effects of a weakened affinity of

haemoglobin to bind O_2, reduced O_2 solubility in the water and the impaired cardiac performance as ambient temperature increases (Farrell, 2002, 2007; Pörtner, 2010). At raised temperatures oxygen utilisation/uptake by somatic tissues is increased depleting blood oxygen levels and together with diminished haemoglobin affinity and oxygen solubility it may cause the onset of myocardial hypoxia, which weakens contractile function of the myocytes (Steffensen and Farrell, 1998). This may result in diminished cardiac output and ultimately reduced AS (Farrell, 2002, 2007). In a similar manner, when temperature approaches the lower thermal limit, energy generation in the form of ATP becomes inefficient, which may impact the level of performance and ultimately survival (Pörtner, 2010).

Seasonal changes in AS have been often reported for many adult fish species in association with spawning (Evans, 1984; Adams and Parsons, 1998). Seasonal variation in aerobic capacity has also been observed in immature fish (Chipps et al., 2000; Yan and Xie, 2011), where factors other than gonadogenesis (generation and development of the reproductive organs) should be responsible for the change. It has also been shown that metabolism can oscillate independently of acclimation temperature, therefore thermokinetic responses alone may not be able to accurately describe annual aerobic demands (Evans, 1984). Temperate dwellers throughout annual cycles experience temperatures where individual and population fitness is optimised and conversely when they may face extreme temperatures associated with reduction of aerobic capacity often observed in effects on growth performance (Clark et al., 2013; Farrell, 2016). However, numerous fish species possess the capacity for metabolic thermal compensation, which is an ability to acclimate to maintain activity of physiological functions and metabolic rates irrespective of change in environmental temperature (Clarke, 1983; Angilletta, 2009; Schulte et al., 2011). The process of acclimation differs among species and depending on the scale of rate of temperature change it may need from a few days to a few months to establish (Sandblom et al., 2014). Three strategies that may solely or in combination be employed by fish to minimise temperature change effects were proposed: quantitative strategy via changing enzyme concentrations and reactions; qualitative via change in protein (enzyme) isoforms; and modulation via altering protein milieu (Clarke, 2003). Therefore temperate fish may exhibit full thermal compensation (e.g. Shorthorn sculpin, *Myoxocephalus scorpius*, Sandblom et al., 2014), if metabolic plasticity is present in their genetic makeup, partial compensation (e.g. southern catfish, *Silurus meridionalis*, Yan and Xie, 2011) or no compensation (Atlantic killifish, *Fundulus heteroclitus*, Healy et al., 2017) when behavioural mechanisms are employed to ensure that their position is maintained within borders of their optimal thermal niche, or any combination of the above (Jobling, 1997). However, the physiological mechanisms and dynamics of thermal acclimation in regulating aerobic scope still need to be systematically investigated (Franklin and Seebacher, 2009; Healy and Schulte, 2012).

3.1.4 Temperature effects and Q_{10} effect

Increases in rates of biochemical reactions or reaction kinetics vary with temperature according to the Boltzmann factor or its recognised approximation called the Q_{10} effect, which is the increase in the rate of the activity with every 10°C increase in temperature (Gillooly et al., 2001; Dillon et al., 2010). The Q_{10} effect is observed because an increased temperature enhances molecular kinetics and enzymatic activity and results in an exponential increase in cellular respiration (Sandblom et al., 2014). Q_{10} values typically range between two and three and is clearly evident when resting metabolic values are compared between fish from polar and tropical regions, as has been demonstrated in Clark and Johnston (1999). They showed that on average polar fish at 0°C need six times less O_2 to support their resting metabolism than tropical fish at 30°C. In other words, oxygen demand needed to sustain metabolic activity increases with temperature, which ideally should stay within the thermal tolerance zone of a given species (Clark and Johnston, 1999). Q_{10} effect as an expression of sensitivity of a function to temperature change is useful when a level of thermal compensation is examined. If thermal compensation occurs during acclimation process, Q_{10} for a rate function compared for two acclimated temperatures will normally reduce to values between 1.0 (perfect thermal compensation) and 2.0 (Farrell, 2016).

3.1.5 Objectives of the chapter

By knowing basic aerobic demand (i.e. RMR) and the maximum level of metabolic activity that is still supported by aerobic processes (i.e. MMR), both encompassing a capacity or potential that could be utilised simultaneously amongst physiological and behavioural activities, evaluation of how plastic fish are in the face of environmental challenges and consequently where they are placed on the survive – thrive gradient can be carried out.

Two coastal temperate test species, snapper and yellow eyed mullet, have distinct growth strategies (chapter 2) that may reflect different levels of basic aerobic needs and maximum aerobic capacities, as well as different compensatory mechanisms in relation of ambient temperature change. To support fitness optimisation in seasonally changeable environments the size of AS may be responsible for growth performance, or other intrinsic or extrinsic factors may play a more pronounced role in test species wellbeing and fitness.

Therefore, the aim of the chapter is to characterise the metabolic phenotype of the test species as a function of temperature, by measuring resting and maximal MO_2, including the sustainable swimming capacity U_{crit}, and finally by determining aerobic scope at acclimation temperatures of 13, 17 and 21°C,

that correspond to the temperature range commonly experienced in their native environments. In addition, Q_{10} effects were calculated to determine metabolic temperature dependence and level of thermal compensation exhibited by the test species.

3.2. Material and Methods

Work on the metabolic activity of snapper was carried out at two different locations. The first part took place at the University of Canterbury. This encompassed all initial respirometry work at 17°C which included the determination of resting (i.e. routine, RMR) and maximum metabolic rates (MMR) as well as specific dynamic action (SDA) and its determinants for a set of different rations (for details about SDA work see chapter 4). The second part was conducted at the Plant & Food Research (PFR) facilities in Nelson (293 Akersten St. Nelson, 7010). There, the rest of the work on snapper (RMR, MMR and SDA at 13 and 21°C) was performed. At the same facilities the entire respirometry work on YEM took place (RMR, MMR at 13, 17 and 21°C and SDA at 17 and 21°C). This is mentioned since there were slight differences in the experimental set up and protocol between the two sites. However, extra care was employed when switching from the University of Canterbury to the research facilities in Nelson, to ensure that data obtained were within the limits that had been established at the University.

3.2.1 Respirometry – Experimental set up and protocol at University of Canterbury

3.2.1.1 Acquisition of experimental animals and husbandry procedures

Snapper originated from PFR research facilities. They were hatched as a part of a breeding programme intended for research or restocking purposes produced by mixed brood stock (i.e. reared and/or captured brood stock). Fish of approximately 100 g were lightly anaesthetised (Appendix 1) in 10 ppm AQUI-S™ (AQUI-S, New Zealand LTD, Lower Hutt, New Zealand) in the resident 5000 L tank. After reaching stage *1* anaesthesia fish were placed in 20 L plastic bags containing approximately 10 L of 50-50% mix of fresh and 10 ppm AQUI-S™ sea water. Bags were subsequently filled with compressed air and sealed, sited in a plastic transporting container and shipped via air for approximately 40 minutes flight duration to Christchurch (the city where the University is located).

On arrival at the University fish were put into 1500 L recirculating tanks set to 17°C in the central resident aquarium and kept in low density conditions (app. 2–5 kg 1000 L^{-1}). The recirculating tank array (two by two tanks) system is equipped with a protein skimmer and bio-filtration system ensuring

high water quality. In addition, twice a week water from the tanks was replaced with a fresh supply. Water quality was regularly tested for ammonia, nitrate and nitrite levels with API (Aquarium Pharmaceuticals, Mars Fishcare, North America) test kits. Experimental fish spent at least 1 month in the home aquarium before they were assigned to any experimental procedures. The fish were fed once every other day to satiation with various fish fillets (e.g. hoki – *Macruronus novaezelandiae*, spiny dogfish – *Squalus acanthias*, silver trevally – *Pseudocaranx dentex*) or green-lipped mussel (*Perna canaliculus*). Water temperatures in the recirculating tanks can be regulated individually, so for the purpose of this part of the study, fish were acclimated and kept at 17°C.

3.2.1.2 Temperature – Controlled room set up

All experiments conducted at the University took place in a temperature controlled (TC) room. The TC room was arranged as a replica of the main aquarium in such a way that all relevant physical conditions were matched between the two settings. This included water temperature, light intensity and photoperiod (day-night light cycling). Light intensity was first measured in the resident aquarium with LI-192SA Underwater Quantum Sensor (LI-COR Environmental Division, Lincoln NE 68504). The intensity in the resident aquarium was 0.05 μmol s^{-1} m^{-2}. The same was established in the TC room by the adjustment of the light source and with the use of neutral density filters. Day-night photoperiod was set to match the main aquarium with 12 hours night (beginning at 7pm) following 12 hours light (beginning at 7 am). In addition to this a light dimming system was employed in the TC room to minimise effects of an abrupt light change (from dark to light and vice versa), which could affect levels of oxygen consumption (MO_2) due to induced stress.

A closed-box respirometer was placed in each of two 1000 L tanks (dimensions 120 x 120 x 70 cm) and the tanks were filled with approximately 350 L of fresh seawater to just cover the lid of the respirometers. Preliminary testing of the water quality was conducted before a full experimental set up was established in the following manner: A snapper was kept in a respirometer as per the experimental protocol (see 3.2.3.1). The system was running for several days and water quality was frequently monitored for any elevations in ammonia, nitrate and nitrite. Elevation in nitrogenous waste appeared to approach critical levels (0.5 mg L^{-1}; Dolomatov and Zukow, 2011) after approximately 3 days (~72 hours). Therefore, a complete exchange of water in the two experimental tanks was practiced every other day and regular monitoring of the water quality ensured that it was kept within the required boundaries. Water temperature was regulated to 17°C ± 0.3 °C.

3.2.1.3 Closed-box respirometer used for respirometry with intermittent flow

The type of respirometer used during the course of the thesis was a closed-box respirometer. The box was built from polyvinyl-chloride (PVC) pipe covered with a transparent acrylic (Poly-methyl methacrylate) lid. The capacity of the respirometer was 6.78 L (dimensions – 155 mm height, 236 mm diameter). This size was sufficient for the biggest fish used in this study (approximately 177 mm fork length) to securely turn about its own axis. On the acrylic lid four holes were drilled, two to accommodate fittings of inlet and outlet water tubes, one for an oxygen electrode in tightly fitting rubber bung to seal the respirometer, and one for a cable connecting a small submersible aquarium pump (model 06302, 378 L h^{-1}; TMC, Taiwan). The purpose of the pump was to ensure uniform distribution of dissolved O_2 in the respirometer at all times regardless of levels of fish activity. In the 1000 L experimental reservoir the same type of pump was also installed. Its function was to pump freshly aerated water into the respirometer in exchange for the O_2 depleted water from fish respiration (Fig. 3.1). The water in the 1000 L tanks was under constant vigorous aeration, which ensured nearly 100% O_2 saturation in the reservoir tanks at all times.

3.2.1.4 Hardware and software data acquisition set up for respirometry with intermittent flow

When a fish was placed into the respirometer, the lid was firmly screwed and the O_2 electrode was fitted in its opening. The O_2 electrodes used were Microcathode Oxygen Electrodes by Strathkelvin, model 1302 (Strathkelvin Instruments, Glasgow, Scotland). The electrode was connected to a compatible oxygen meter, model 781 (Strathkelvin Instruments, Glasgow, Scotland). The meter was further connected to the data acquisition device PowerLab T-26 (A.D. Instruments, Waverly, N.S.W., Australia). The PowerLab was equipped with two distinct outputs. One output led to the computer where an electrical signal representing level of dissolved O_2 was converted via the compatible data acquisition software (LabChart 7 v7.3.8., A.D. Instruments) into units of choice (mmHg, millimetres of mercury) and recorded. The software was programmed to send two different commands back to the PowerLab when the O_2 tension in the respirometer reached two predetermined levels. First, due to fish respiration, O_2 tension would decrease to 120 mmHg (approximately 80% O_2 saturation). Subsequently the recurrent signal from the computer was sent back to the PowerLab and via its second output to a solenoid. The solenoid opened the valve, which actuated water exchange between the reservoir tank and the respirometer. Soon after (within 2–3 minutes) the software recognised that ascending O_2 tension had reached 140 mmHg (approximately 93 % O_2 saturation). At that point the software would send another signal back via the PowerLab to the solenoid conveying a command to close the valve, which would cause the flushing to cease. The process was repeated as long as the fish was required to be in the respirometer. The LabChart-PowerLab-solenoid feedback loop could have been overridden at

any time with the manual switch. Furthermore, as a precaution, in the instance of hardware and/or software malfunctioning the solenoid was wired to automatically open the valve, which would ensure the fish remained unharmed. These measures were in place to ensure that the fish never encountered low oxygen/hypoxic conditions in the respirometer during recording sessions.

3.2.1.4.1 Oxygen electrode calibration and correction for effects of barometric pressure

Before putting a fish in the respirometer, the oxygen electrode had to be calibrated in order to accurately measure the change in O_2 tension. Two-point calibration with 100% O_2 saturated sea-water (produced by vigorous air bubbling) and 0% saturated seawater (by partially dissolved 10 mg ml^{-1} crystaline sodium sulphite, Na_2SO_3) was performed. Furthermore, since change in barometric pressure affects the rate at which oxygen enters the water from the atmosphere, the following equation was employed to correct for this effect: $PO_2 = (BP - WVP) \times 0.2094$. PO_2 stands for partial pressure of oxygen in the 100 % oxygen saturate water (mmHg), *BP* is the barometric pressure (mmHg), *WVP* is the water vapour pressure at the given temperature, and *0.2094* is the fraction of oxygen present in the atmospheric air.

3.2.1.5 Data processing

The metabolic rate or energy expended by a fish during the experimental procedures in this thesis are expressed as mg O_2 kg^{-1} h^{-1}. MO_2 (measured as O_2 tension depletion in a known body of water) is recorded in LabChart software in mmHg, which is a monometric unit of pressure. To convert values from mmHg into mg O_2 kg^{-1} h^{-1} the following equation was utilised:

$$\frac{\Delta PO_2 \times C \times V \times 31.999}{t \times M}$$

ΔPO_2 – difference in oxygen partial pressure between two measurement points (mmHg); C – oxygen capacitance (solubility of oxygen in water) of seawater at a given temperature (µmolL^{-1}mmHg^{-1}); V – volume of water in the respirometer (L); *31.999* = molecular mass of oxygen (g mol^{-1}); t = time interval of a measurement (h); M = mass of a fish (g).

3.2.1.5.1 The principle of data acquisition

When O_2 tension depleted in the respirometer, the LabChart software depicted the event as a line forming a negative slope. The beginning of the slope corresponded with the closing of the solenoid valve and the moment when the slope changed its direction from downward to upwards, corresponded with the opening of the valve. The difference between the beginning and end of the negative slope represented ΔPO_2 within the elapsed time t from the above equation. The duration of the elapsed time t varied between 10 to 45 minutes depending on the fish's demand for the oxygen. For the calculation of SDA parameters MO_2 for a given hour was calculated as an average of all measurements that were recorded within the hour. However, using the exact point in time when the solenoid closes the inlet to calculate the ΔPO_2 is not recommended, since a time is needed for a probe to readjust to change in oxygen pressure and for water to be properly mixed in the respirometer (Svendsen et al., 2016). Therefore, the starting measurement for the calculation of ΔPO_2 was considered to be ~1 minute after the shutting of the solenoid valve, when the slope had started to decline linearly.

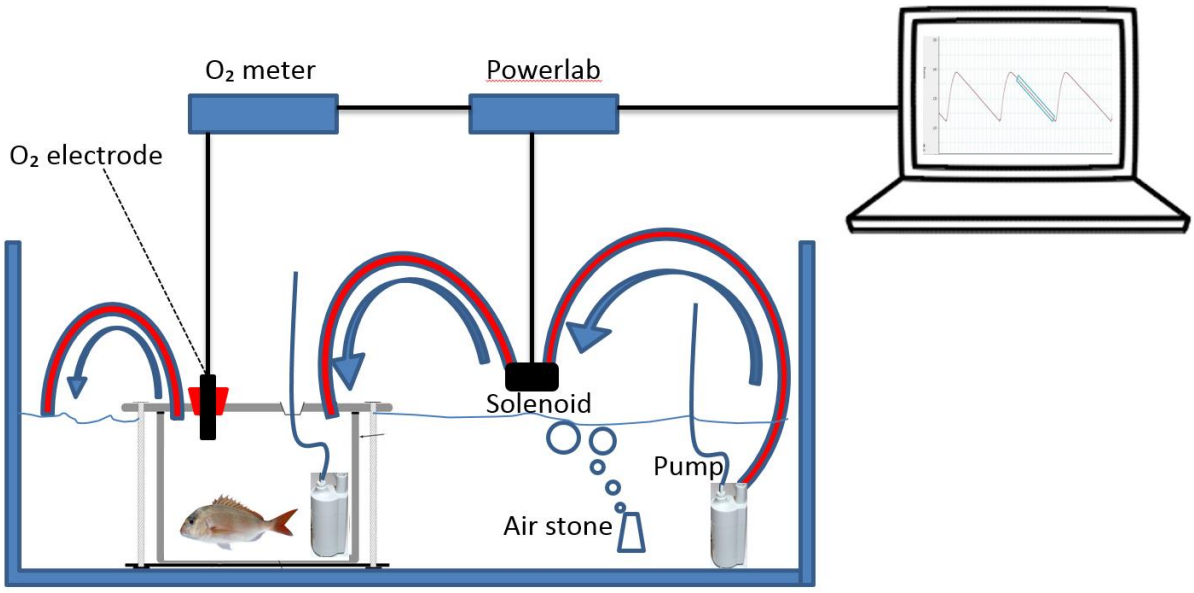

Figure 3.1. Schematic illustration of closed-box respirometer with intermittent flow set-up at the University as described in 3.2.1.3 and 3.2.1.4. The blue arrows represent the direction of water movement, red represents the tubing set up of the system.

3.2.2 Respirometry – Experimental set up and protocol at PFR facilities

3.2.2.1 Acquisition of experimental animals and husbandry procedures

3.2.2.1.1 Snapper

Twenty–five snapper were provided by PFR directly from rearing tanks at the facility. Fish were kept in an 800 L flow-through tank (~ 20 L min^{-1}) connected to a temperature controlled 7000 L silo supplied with sand and UV filtered seawater pumped directly from the Nelson Haven. The silo was equipped with four titanium HX-8 heat exchangers (Vaportec Spirex, NZ) and an aeration system (four AQS385 air diffusers, Aqui-S, NZ) to ensure good maintenance of selected temperature and proper mixing all through the silo. When the selected temperature in the silo was too low or too high for the requirements of this study, an additional 2 kW (model NEMA 4X, Agua Logic, Inc., San Diego, CA) aquarium heater was used or the water supply was switched directly to the source of the ambient water. Additional aeration was also provided in the tank. The tank was cleaned weekly and the feeding regime was the same as at the University.

3.2.2.1.2 YEM

In early February 2016, when the ambient sea-water temperature in Nelson Haven was ~20°C, 60 YEM ranging from 90 to 140 mm fork length (FL) were obtained from the wild. They were caught with a Monorail dip net (40 cm x 40 cm, 6mm mesh, 152 cm handle; Auqasonic, Wauchope, NSW) at the seawater outlet coming from the flow-through seawater tank system at the PFR research facility. The drained water is enriched with organic matter from the fish tanks and at the outlet it attracts hundreds of opportunistic feeders like YEM. Within a few attempts YEM were scooped with the dip net and placed in a 20 L bucket then taken to an 800 L tank set at 21°C to acclimate. The 800 L tank was on the same water supply line as the snapper tank. The other husbandry procedures were the same for YEM as for the snapper.

3.2.2.2 Acclimation

Twenty–five snapper were first introduced to the 800 L tank, set to 21°C, in mid-February 2016 from a resident tank supplied with an ambient sea-water with temperature of ~20°C. At that time YEM was already acclimating to the captive environment. All fish were required to be eventually acclimated to three experimental temperatures – 13, 17 and 21°C. This temperature range is likely to be experienced

by fish in Nelson Haven; however, the spectrum does not encompass temperature extremes that the dwellers in the area may face on the yearly basis (i.e. ~9–23°C). The reason for the selection was not only to mimic the thermal conditions of the area, but also to accommodate requirements of SDA work (for details see chapter 4). Nevertheless, whenever possible, acclimation was done in a fashion to utilise natural seasonal decrease and increase in ambient sea-water, which imposed minimal effects on fish physiology while they were acclimated to the desired temperature. When this was not possible, acclimation was proceeded at the rate of approximately 1°C day^{-1} by means of gradual water temperature increase/decrease via mixing of ambient and heated silo's water and/or utilisation of aquarium heaters. If this method was used, once the required temperature was reached, fish were kept, on average, an additional 3–4 weeks before onset of experiments. YEM spent at least 2 months at first acclimated temperature (i.e. 21 °C) before they entered respirometry trials.

3.2.2.3 Respirometry room set up

All respirometry work at the PFR facility was conducted in the respirometry room. The room was supplied with flow-through seawater with the option to come from a temperature-controlled silo or from the system that supplies ambient water directly from the Nelson Haven. The most suitable water supply would be used as required. In addition, seawater would pass through a series of plate heat exchangers connected to a Grant LTD 6/20 heated circulating water bath (Grant Instruments, Cambridge, UK) with Grant LTD 20G heat unit (1.4 kW heating and 0.56 Kw cooling capacity). The system was set up so that the water temperature did not exceed more than ± 0.2 °C of the required temperature. The same closed-box respirometers as at the University were utilised at PFR facility. Both respirometers were placed in an empty 300 L tank that served as their holding space and as a physical and visual protection for fish rather than a water reservoir as was at the University. This was because respirometers were connected directly to the seawater supply when it passed through the heat exchange system and the flow meter. In addition, outlet tubes connected respirometers with the drainage. In the outlet tubes, a temperature probe was installed for additional monitoring of the water temperature. For water mixing purposes, a different set of pumps than at the University were installed in the respirometers. However, the performance was the same since Mi Mouse submersible aquarium pump (SICCE, Vicenza, Italy) has an adjustable flow option which was set to match the strength of the TMC pump used at the University.

Light, temperature and space conditions were set up to match the conditions established at the University. To match the light conditions a portable light dimming system, with a timer and light intensity control, was installed in the room. Preliminary work that followed the respirometery room

set-up showed that MO_2 output was preserved between the two sites for the same size fish at the same temperatures.

3.2.2.4 Hardware and software data acquisition set up for respirometry with intermittent flow

The same LabChart-PowerLab-solenoid feedback loop was set up to control the activity of the solenoid and to assist in data acquisition as at the University. The difference was in how the LabChart software was programmed. At the University the solenoid would open or close the valve when O_2 tension in the respirometer descended to 120 mmHg or ascended to 140 mmHg respectively. At the PFR's respirometry room the loop would function across the following commands: *Flush* (6 minutes, the valve is opened) – *Wait* (45 seconds, the valve closed) – *Measure* (16 minutes, the valve closed) circuits. The system suited the overall set up constructed from the incoming seawater plumbing system, heat exchangers, flow meters and the solenoid array connected through 16 channel PowerLab (model 16/30 880) to LabChart Pro (v8.0.6). Measurement of MO_2 was undertaken as at the University, with data averaged across a one hour period to determine the hourly metabolic rate. Measurements conducted in this fashion generally did not allow fish to deplete oxygen below the 120 mmHg (approximately 80% O_2 saturation) threshold.

3.2.2.4.1 Introduction of Fibre-Optic technology

During the course of the study Fiber-Optic Oxygen Sensors OXROB10 and OXROB3 with FireStingO_2 USB-powered fibre-optic oxygen meter (PyroScience GmbH, Aachen, Germany) were introduced instead of the Strathkelvin O_2 tension measurement set-up. An associated software, FireSting Logger (v 2.356, Firmware 2.13) processes the signal that is generated after oxygen from the water collides with indicator film on the sensor tip, and displays the activity in selected units in the same sloping fashion as in the LabChart. Since LabChart is not compatible with the FireSting technology two software were merged to work in unison in a way that the solenoid was still governed by the *Flush-With-Measure* loop from LabChart but the actual data recording was performed by the FireSting Logger. The FireSting Logger was set to record data directly to an Excel sheet where the calculation of MO_2 was performed when output of the *Flush-Wait-Measure* loop system from the LabChart was combined.

3.2.2.5 Microbial background respiration (BR), sensors/electrodes drift and respirometer effects

Regular clean-up of the respirometry system ensured minimal impact of BR on MO_2 readings. Before every set of trials the entire respirometry system (respirometers, submersible pumps, associated tubes and pipes, heat exchangers, solenoids and flow meters) were thoroughly cleaned, assembled together and flushed with >11.5 g L^{-1} sodium hypochlorite (concentration 10 mL L^{-1}) solution. The solution was left in the system for several hours then extensively rinsed with fresh water. After the clean-up recorded BR was virtually undetectable and thus considered to be zero. For 24 hours after completion of an individual trial (up to 7 days), when a fish was removed, BR was recorded again and the difference calculated. If it was observed that BR affected more than 1% of the average hourly MO_2, the appropriate fitted line/curve was constructed and its equation used to correct the recorded data. In cases when determination of MMR was taking place BR was measured three times – before, during an exhaustive exercise test (as a fish was not in the respirometer at that time), and after the experiment. Within a set of trials, before any new fish was used the system was vigorously rinsed with fresh water and BR check was routinely performed again. BR was negligible at 13°C; however, small corrections were required at 17 and 21°C.

All types of oxygen sensors used in this study were also subject to drift testing. The drift testing was performed during the first 24 hours (FireSting optic sensors) or 48 hours (Strathkelvin electrodes) in a fully operational system in absence of fish when the BR was considered to be zero. If during that period a deviation from the straight horizontal line occurred that could not be explained by change in water temperature or barometric pressure it was considered as sensor drift. In the same way as BR, the drift was dealt with and corrections to recorded data were made.
Preliminary work suggested that there were no effects of respirometer used in the respirometery work on recorded data, therefore the factor *respirometer* was not included into any model used to analyse MO_2 data either for determination of metabolic rates (this chapter) or specific dynamic action (chapter 4).

3.2.3 Measurement of RMR, determination of sample size and metabolic scaling

3.2.3.1 Measurement of RMR at the University

Before commencement of an experiment fish fasted for 2 days (~48 hours), which was sufficient time for the digestion to cease, as required for determination of resting and subsequently specific dynamic action (SDA) data (Secor, 2009; Chabot et al., 2016). Individual fish were first lightly anaesthetised in

75 mg L^{-1} MS-222 (Appendix 1). Fork length and mass was taken as described in chapter 2 and the fish was placed in a bucket with fresh (no anaesthetic) sea-water to recover. Subsequently the fish was taken to the TC room and placed in a respirometer and left to settle in the new environment. Recording of MO_2 commenced immediately. The initial data were inspected to observe the level of stress induced by handling and anaesthesia. The monitoring continued for as long as fish needed to fully recover and start exhibiting constant resting stage (i.e. when recorded MO_2 data dropped to the lowest level after fish was introduced in the respirometer and were kept on average at that level for at least four consecutive hours). Generally, fish would reach this stage within 24 hours, but some fish needed up to 48 hours to be fully rested.

Data that were recorded for the period of 24 hours after the resting stage had been reached was considered as RMR of a given fish. After the resting data were recorded maximum metabolic rates (MMR), and SDA responses were measured.

3.2.3.2 Measurement of RMR at PFR facilities

3.2.3.2.1 Snapper

At the facilities the following parameters were determined: snapper RMR at 13 and 21°C, and YEM RMR at all three experimental temperatures. All procedures for obtaining RMR data at PFR were performed in the same order and fashion as at the University.

3.2.3.2.2 YEM, preliminary respirometry trials

YEM as a fish with a schooling lifestyle was expected to be less motivated to take a meal when alone in a respirometer. Therefore, the objectives of preliminary trials were to establish the conditions that allowed an individual YEM to consume a meal in a respirometer. As expected individual YEM refused to eat; however, when a group of YEM were placed in the respirometer meal consumption was instant. Therefore, it was decided that mimicking a group of YEM with a set of mirrors should be tested. The mirror trials were carried out at 21°C with a successful outcome. The mirror arrangement that produced the quickest feeding response was accepted and kept through the all respirometry work. The respirometry work on YEM was more challenging and time consuming than snapper, hence the sample size was arbitrarily dropped to eight. All procedures for obtaining YEM RMR were the same as for snapper.

3.2.3.3 Determination of the sample size for calculation of RMR

All fish that eventually ended up in SDA experiments were initially used to produce RMR. RMR data sets calculated for a consecutive 24 hours served as a baseline curve against which SDA was calculated. Therefore, apart from RMR being obtained for each of three experimental temperatures it was also estimated for all levels of the ration treatment (i.e. 0.5, 1, 2 and 3% and 0.5 and 1% body mass (BM) ration for snapper and YEM respectively in chapter 4). The effects of the ration size on SDA was conducted only at 17°C. Consequently, the sample size for test species RMR at 17°C was averaged out of all fish that produced a full set of 24 hours resting data (Table 3.1).

3.2.3.4 Metabolic scaling to common mass

For the purpose of comparing metabolic rates of two test species, metabolic scaling to the common mass of 100 g was performed. Scaling can be explained as the structural and functional outcomes of alteration in size amongst similarly organised animals (Schmidt-Nielsen, 1977). In particular, body mass has a profound influence on the metabolic rate, where generally, larger organisms use more O_2 than smaller. However, the direction of relationship is reversed when it is observed on a mass-specific basis, in which case smaller animals have higher rates of O_2 consumption. This well-established pattern can be expressed with the equation $Y = aM^b$, where Y is resting or maximum metabolic rate, a is the mass coefficient, X is the mass of the animal, and b is the scaling exponent/coefficient or slope of the allometric plot of log Y as a function of log M. Coxon (2014) provided scaling coefficient for both RMR (0.74) and MMR (0.81) for snapper; however, there were no published examples for YEM RMR and MMR. The closest work relative to YEM that generated the RMR exponent was produced by Bin and Xian (2005) where the subject redlip mullet (*Liza haematocheila*) exhibited mass scaling for the resting metabolism at the power of 0.84. This value was utilised for the scaling of YEM RMR. Furthermore, literature suggests (e.g. Killen et al., 2007; Huang et al., 2013; Norin and Clark, 2016) that the scaling exponent is generally higher for MMR than for RMR, likely owing to volume-related muscular power production (Glazier, 2005, 2009). On average an increase of scaling coefficient from RMR to MMR was found to be ~7% (Glazier, 2009; Killen et al., 2007; Huang et al., 2013). This increase factor was employed to estimate the scaling exponent of YEM MMR, hence 0.90 was adopted for the purpose.

3.2.4 Determination of maximum metabolic rates (MMR)

3.2.4.1 MMR via exhaustive aerobic exercise

Exhaustive aerobic exercise was the vehicle for fish to exhibit MO_2 regarded as MMR. A single fish was brought after a series of water current speed increments to the exhaustion point when it lost ability of swimming and keeping its position in the water column. At this point it was considered that the fish reached the maximum level of aerobic activity or MMR. An important side product of the exercise was a critical swimming speed (U_{crit}). U_{crit} is a standard measure to express sustained swimming capabilities of fish (for details see 3.2.4.3).

The exercise was carried out in the Blazka style swimming flume (Fig. 3.2). The flume was constructed of 80 L transparent acrylic tube (70 cm long and 20 cm in diameter) bounded with plastic mesh at both ends to prevent fish from getting injured during the exercise. The flume is positioned inside of a 125 cm long acrylic box. The box sits on a metal frame with a motor underneath. The motor is in charge of running an impeller that is connected to the flume at one end. As the impeller rotates a water current is established against which fish swim. The speed of the current is calibrated so that a number of impeller rotations (Hz, rotations per second) represents a known water velocity. The common approach of presenting swimming speed of fish is to use length standardised values expressed as body lengths per second (bl s^{-1}). From previous work done on snapper it was expected fish would swim up to 7 bl s^{-1}. Therefore, for each individual fish a table with impeller rotation values (Hz) corresponding to water current velocity of 0.5-7 bl s^{-1} was produced. When a fish was first introduced to the flume it was left to acclimate to the new environment (change in light conditions and an introduction of constant vibrations produced by the running machine) for a period of 30 minutes with a water current speed at 0.5 bl s^{-1}. After the initial 30–minute orientation period the fish was submitted to a formal exercise experiment by increasing the current speed to 1 bl s^{-1}. The fish swam at this speed for a 15–minute period after which the speed was increased to 2 bl s^{-1}. The 15–minute increments continued until the fish stopped swimming and fell back against the rear mesh of the swimming tunnel. At that moment the machine was instantly switched off and the fish was as swiftly as possible transported to a respirometer in order to capture the level of MO_2 representing its MMR. The principle of MMR determination outlined here was previously described in several other studies (e.g. Kolok, 1999; Coxon, 2014).

Generally, there were no issues with snapper during the exhaustive swimming exercise. However, as mentioned earlier working with YEM required extra precaution in designing experimental procedures. Similar to refusal of food in the respirometer, individual YEM would not regularly perform in the swimming flume. Moreover, even when handling (e.g. transporting fish from the respirometry room to the exercise machine in a white 20 L bucket filled with ~5 L sea-water) was not carried out with extra

care YEM would exhibit behaviour indicating high levels of stress (i.e. vigorous, erratic swimming with occasional attempts to escape/jump out). This all may have contributed to fish inconsistency in swimming. The success of having mirrors in the feeding trials inspired the idea to place mirrors around the flume. This created the visual impression of conspecifics and minimised stress induced by the "outside world" through the clear flume walls. The set-up markedly increased YEM swimming performance especially at 13 and 17°C, and somewhat at 21°C. The original intent was to generate MMR via exhaustive exercise in the swimming flume for 10 fish of each species at each of the three experimental temperatures. However, an alternative approach, a five minutes stick bucket chase, had to be introduced because the original design did not work perfectly for all fish and temperatures (see 3.2.4.2). This reflected the final sample sizes for MMR for the two test species (Table 3.1).

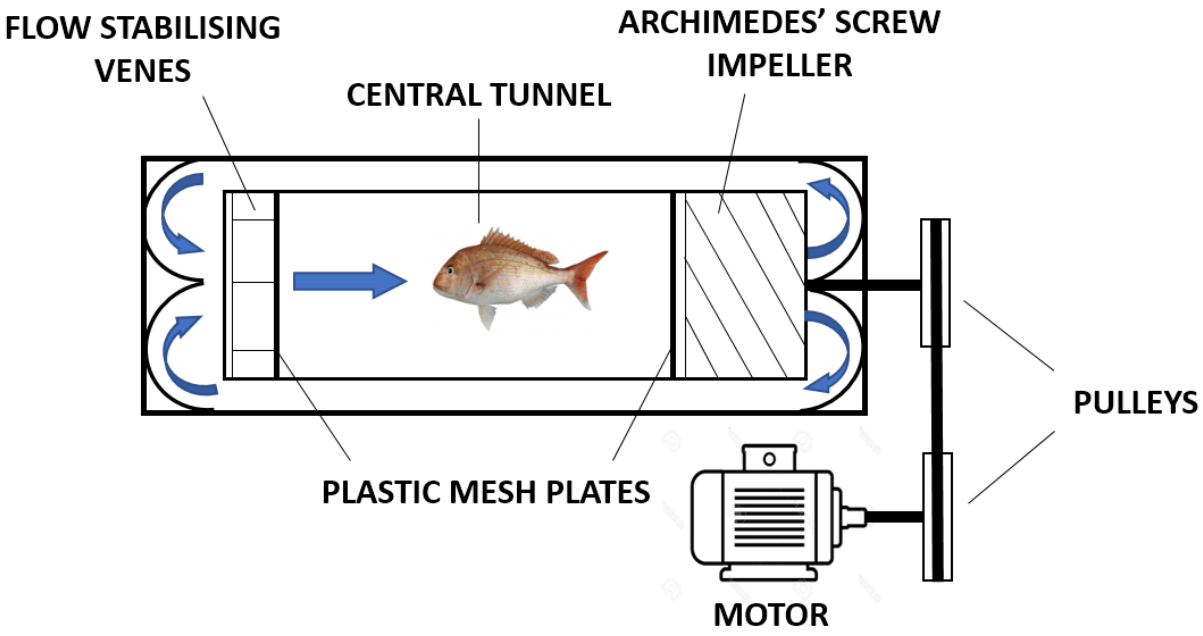

Figure 3.2. Schematic illustration of Blazka-style swimming flume as explained in 3.2.4.1.

3.2.4.2 Alternative approach for obtaining MMR, the five minutes bucket chase

Rarely fish were not willing to swim in the flume and the alternative approach, which was to vigorously stick chase those fish in a bucket for a period of 5 minutes, was employed (Soofiani and Priede, 1985; Reidy et al., 1995; Clark et al., 2012; Clark et al., 2013). This approach did not work for both test species equally. The MO_2 rate produced by snapper after the bucket chase was markedly lower than average MO_2 produced via the swimming flume. Therefore, the bucket chase data were not included in final MMR calculations for snapper. The mirror set up around the swimming flume for YEM did not work well at 21°C. Therefore, at that temperature YEM were also submitted to the 5–minute bucket chase. Contrary to snapper, MO_2 values produced after the chase were not different to those produced after exhaustive exercise, hence they were pooled together to generate final YEM mean MMR at 21°C.

3.2.4.3 Critical swimming speed (U_{crit}) determination

U_{crit} is a parameter generated after assessing maximum sustainable swimming speed of a fish under the laboratory conditions (Brett, 1964). During the exhaustive exercise the moment when a fish lost ability to perform in the water column was also the final point required for calculations of U_{crit}. For the calculation the following equation was utilised (Hammer, 1995):

$$Ucrit = Vs + \left(\frac{Te}{Ti}\right) Vi$$

Vs – Second to last speed (bl s^{-1}) before fish collapsed
Te – Time elapsed within the final swimming speed at the moment fish collapsed
Ti – Time interval between two swimming speeds
Vi – Speed increment

For sample size, mean mass, length and standard deviation of fish involved in producing U_{crit} see Table 3.1.

3.2.4.4 Determination of MMR and fish recovery

After exhaustive exercise fish were transported to the respirometry room, placed into a respirometer and as soon as it was sealed reading of MO_2 was carried out. On average, time between turning off the exercise machine and beginning of post-exercise metabolic rates measurement would not exceed more

than a minute. MMR was determined within the first 4 minutes of the elapsed recorded time. The 4–minute time was chosen as it was observed that on average this was the length of time required for oxygen sensors to readjust and for the system to record a fraction of the slope in clear linear fashion long enough for accurate calculation of metabolic rates. Coxon (2014) was a source for the adapted practice of monitoring the recovery time for up to 21 hours post-exercise. The same timeframe was also applied for YEM. An individual fish was considered to be recovered when descending level of MO_2 reached the previously determined RMR level of the fish.

Table 3.1. Sample size, average mass and length values with associated standard deviations (S.D.) of fish that produced data for calculation of RMR, MMR and U_{crit} at three experimental temperatures (13, 17 and 21°C).

RMR	Temperature (°C)	n	mean mass (g)	S.D.	mean length (mm)	S.D.
Snapper	13	10	91.5	6.7	164.4	4.1
	17	33	99.7	18.9	166.2	8.4
	21	10	107.5	15.6	167.2	7.6
YEM	13	8	34.0	2.7	150.4	2.3
	17	20	32.8	3.0	151.9	2.8
	21	8	29.4	3.9	144.1	7.0
MMR						
Snapper	13	9	92.6	6.6	164.8	4.3
	17	8	92.0	7.5	161.6	5.8
	21	9	110.3	13.8	168.7	6.5
YEM	13	6	32.8	1.9	149.3	1.6
	17	9	34.4	2.7	153.1	1.2
	21	8	28.8	3.2	144.1	7.0
U_{crit}						
Snapper	13	14	92.0	6.0	165.1	4.3
	17	11	90.1	13.8	161.6	5.8
	21	10	107.5	15.6	167.2	7.6
YEM	13	6	33.4	2.4	149.3	2.1
	17	8	34.6	2.7	153.4	1.3
	21	3	25.9	1.5	137.7	2.4

3.2.5 Statistical methods

3.2.5.1 General overview

All data upon collection were first inspected and processed in Excel as described in chapter 2. The relationship between two variables was tested with the liner regression model in SigmaPlot. To test the effects of a single or two factors on multiple groups one-way or two-way ANOVA was employed and performed in R. When a covariate was required in an ANOVA model, the use of general linear model ANCOVA was warranted and it was also performed in R. All associated mandatory statistical steps (e.g. assumptions checking, data transformation) were carried out as outlined in 2.2.6.1. Only fish with full sets of data were accepted for the final statistical analysis. Preliminary work demonstrated that there were no effects of respirometer (two respirometers were used simultaneously throughout the respirometery work including metabolic rates and specific dynamic action) on respirometery data therefore blocking factor *respirometer* was not included in any models dealing with the respective data.

3.2.5.2 RMR

- Intraspecific comparison within temperature treatment were tested with the general linear model ANCOVA as presence of fish *mass* data as covariate was required. When there was not observed effect of *mass*, the covariate *mass* was excluded from the model which by default turned into one-way ANOVA

- Interspecific differences as well as evaluation of interaction between factors *temperature* and *species* on the RMR data scaled to the common mass were tested with two-way ANOVA

- Q_{10} temperature coefficient for RMR was calculated according to the equation:

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{10^\circ/(T_2-T_1)}$$

  Where R2 and R1 represent RMR at two temperatures, and T2 and T1 are temperatures at which R2 and R1 were determined respectively.

3.2.5.3 MMR

- Relationship between MMR and fish mass at three temperatures, effects of temperature and species on MMR and Q_{10} coefficient were determined with the same approaches as for RMR.

- Effects of temperature, species and their interaction on recovery time (hours) after exhaustive exercise was tested with two-way ANOVA.

- Effects of temperatures and species on U_{crit} were tested with two-way ANOVA.

3.2.5.4 AS

- Absolute aerobic metabolic scope (AAS) was calculated by subtracting RMR from MMR.

- Factorial aerobic metabolic scope (FAS) was as calculated by dividing MMR with RMR.

- Intra and interspecific comparison of both scopes were performed in the same way as described for RMR and MMR.

3.3 Results

The summary of descriptive statistics and significance testing amongst experimental temperature groups regarding RMR, MMR and AS within and between two test species is outlined in Table 3.2.

For the purpose of visually illustrating and functionally explaining dynamics of variables, all data, wherever permissible, were presented with a curve of best fit representing exponential, natural logarithmic, power or linear model. In cases where none of the mentioned models well explained the dynamics of the data as a function of temperature (i.e. $R^2 \leq 0.85$) the 2nd polynomial function was assigned to graphically illustrate the trajectory of the variable across the experimental temperatures. Since R^2 of 2nd polynomial order assigned to three mean data points is always 1, no equation and R^2 were presented or explained in figures, figure captions or in the text. All error bars in graphs are 95% confidence intervals unless stated differently.

3.3.1 RMR

For the purposes of investigating temperature effects on RMR, 24-hour resting data of a given fish was averaged and as such represented a data point for a given temperature.

3.3.1.1 Snapper RMR – effects of temperature

One-way ANOVA demonstrated significant differences between mean RMR of three groups of data representing 13, 17 and 21°C (Fig. 3.3; $F_{2, 52} = 52.631$, $p < 0.0001$). Furthermore, Tukey pairwise multiple comparisons of means revealed that all temperature groups were different from each other showing RMR significantly increased as temperature increased from 13 to 17 and finally to 21°C (Table 3.2). The relationship of RMR scaling on the temperature vector was best explained with the natural logarithmic model ($R^2 = 0.998$) expressed as:

RMR (MO_2) = -302.19 + 152.40 * ln (temperature [°C]) (Fig. 3.3)

While RMR increased as a function of acclimation temperature the Q_{10} effect declined from 2.75, as observed for temperature increase from 13 to 17°C, to 1.65 for temperature increase from 17 to 21 °C. Overall temperature dependence of RMR between 13 and 21°C had a coefficient Q_{10} of 2.13.

Fig. 3.4 portrays a circadian pattern, as well as the level of variation in RMR of all fish calculated per hour within a 24-hour day. The circadian pattern was overall relatively weak, and variability appeared overall relatively stable for the two lower temperatures, but higher for 17°C. Per hour variability further increased at 21°C, where also relative 24-hour uniformity, as seen for lower temperatures, was lost. This was likely due to the dual night behavioural pattern observed, where approximately 50% of fish in the respirometer at 21°C were relatively quiet compared with the other half, which exhibited extreme levels of activity during the night time (Fig. 3.4, 21°C).

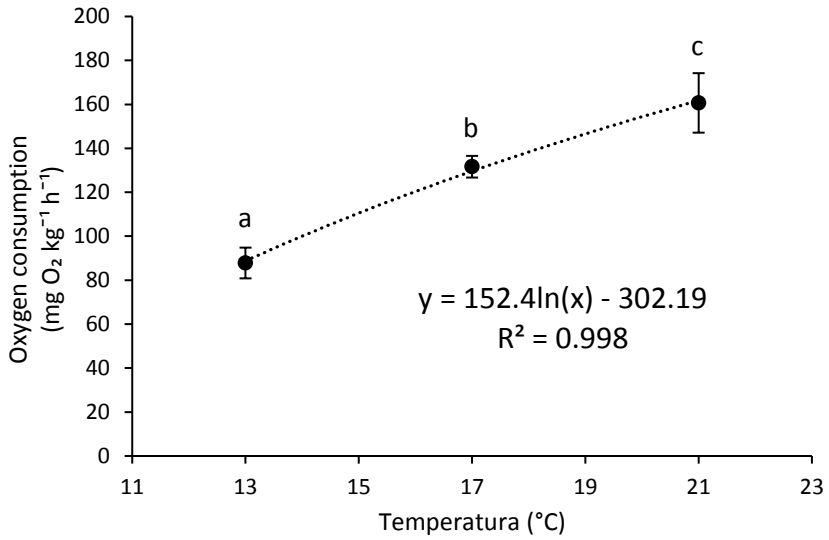

Figure 3.3. Mean mass-specific resting oxygen consumption (routine metabolic rate, RMR, mg O_2 kg^{-1} h^{-1}) of snapper as a function of experimental temperatures (13, 17 and 21°C). Means (black symbols) are connected with a curve of best fit representing the natural logarithmic model with associated equation and R^2. Different lower-case letters denote significant differences in *MO_2* between temperature groups. The caption of other graphs in the chapter are the same as for Fig. 3.3, unless stated differently.

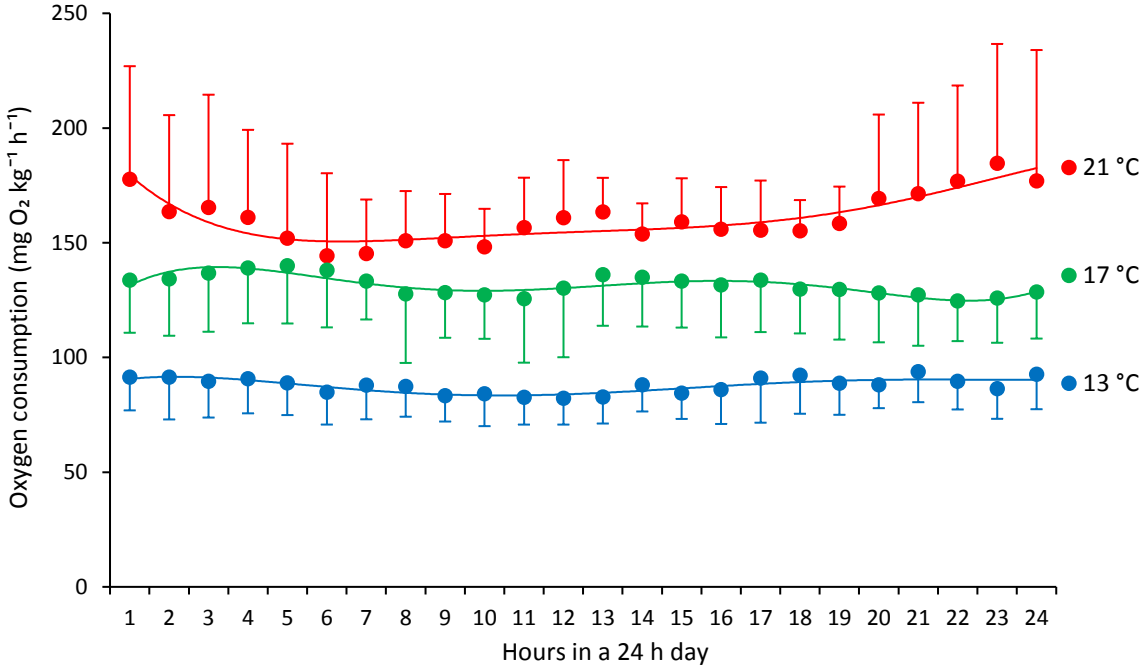

Figure 3.4. Resting *MO_2* as routine metabolic rate (RMR) of snapper for three experimental temperatures (13°C – blue curve and symbols, 17°C – green curve and symbols and 21°C – red curve and symbols) averaged per hour within 24-hour day for all fish that produced a full set of 24 h resting data. Curves depict smoothed circadian pattern in resting data for three temperatures based on polynomial function of the best fit. Error bars are standard deviations.

3.3.1.2 YEM RMR – effects of temperature

The temperature effect on YEM RMR was also evident as differences amongst all three temperature groups were detected ($F_{2, 35}$ = 72.938, p < 0.0001; Table 3.2, Fig. 3.5). As for snapper, YEM RMR scaling along the temperature continuum can be expressed with natural logarithmic function:

RMR (MO_2) = -281.13 + 142.73 * ln (temperature [°C]) (Fig. 3.5).

YEM Q_{10} coefficients were effectively identical to snapper, thus for the range from 13 to 17°C and 17 to 21°C Q_{10} was 2.71 and 1.63 receptively and for the entire range from 13 to 21°C it was 2.10.

Circadian rhythm in YEM RMR was only recognisable at 21°C, when day time activity appeared to be a dominant feature (Fig. 3.6). At 21°C, per hour variability was also notably higher than at 13 and 17°C, when standard deviations were fairly constant for all 24 hours of a day, but still lower at 13°C (Fig. 3.6).

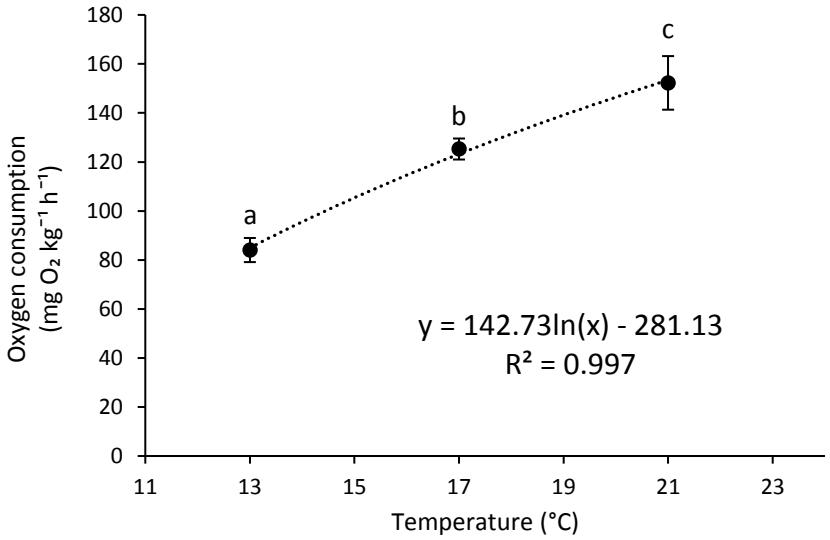

Figure 3.5. Mean mass-specific resting oxygen consumption (routine metabolic rate, RMR, mg O_2 kg^{-1} h^{-1}) of YEM as a function of experimental temperatures (13, 17 and 21°C).

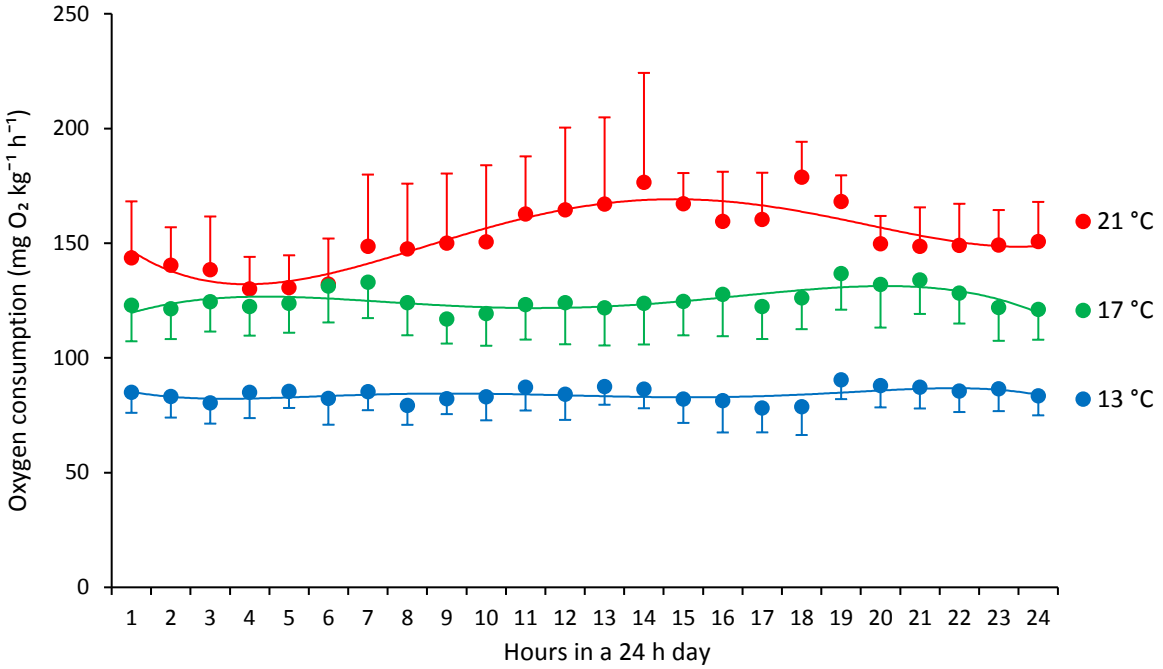

Figure 3.6. Resting MO_2 as routine metabolic rate (RMR) of YEM for three experimental temperatures averaged per hour within 24-hour day. The rest of the figure caption is the same as in Fig. 3.4.

3.3.1.3 RMR Snapper vs. YEM

RMR between the two species differed ($F_{1,\ 88}$ = 67.160, p < 0.0001) for all three experimental temperatures when $\log_{10}$ transformed mean values were compared with their respective temperature counterparts, showed that snapper RMR was always higher than YEM RMR (Table 3.2; Fig. 3.7). The natural logarithmic function was the model that best explained the effect of temperature on the RMR of both species (Fig. 3.7).

In terms of Q_{10} effect between snapper and YEM RMR, standardised data demonstrated that overall YEM were less affected. For the temperature range from 13 to 21°C the Q_{10} effect for YEM was 2.04 while for snapper it was 2.24. Capacity for the thermal compensation was noted for both species only for the temperature increase from 17 to 21°C; however, this was greater for YEM (i.e. Q_{10} effects of 1.55 and 1.75 for YEM and snapper respectively). On the other hand, both species were conforming with a temperature drop from 17 to 13°C, again snapper showing greater thermal effects (i.e. Q_{10} effects of 2.68 and 2.87 for YEM and snapper respectively).

In terms of 24-hour pattern in resting data, at 13 and 17°C, equally for both species, no clear indication of circadian behaviour was observed. Also, per hour variability showed similar pattern, to

generally increase as a function of temperature. At 21°C, RMR of both species demonstrated increased patterns in both, circadian rhythm (i.e. snapper appeared more active during night, whereas YEM during day) and, similarly for both species, variability measured per hour within the 24-hour day was pronounced at the time of the day when fish appeared to be more active (Fig. 3.4, 3.6).

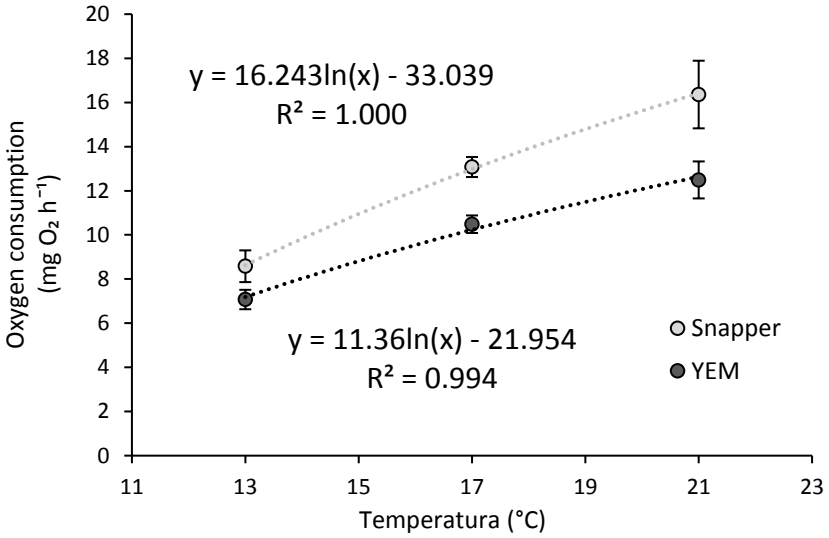

Figure 3.7. Mean values of resting oxygen consumption (routine metabolic rate, RMR, mg O_2 h^{-1}) from data standardised to 100 g of snapper (light grey curve and symbols) and YEM (dark grey curve and symbols) as a function of experimental temperatures (13, 17 and 21°C). Curves connecting the mean values represent the model of best fit (i.e. logarithmic model for the both species), both with their associated model equations and R^2.

3.3.2 MMR

3.3.2.1 Snapper MMR

MMR was measured for an individual fish then averaged for the group representing a given temperature. Effects of temperature on MMR was inspected with one-way ANOVA on ranks and results statistically confirmed differences amongst the temperature groups (H = 17.463, DF = 2, p = < 0.0010, Fig. 3.8). After performing Dunn's pairwise post-hoc tests the difference was revealed only between 13 and 21°C (Table 3.2). Furthermore, unlike RMR, MMR scaling with temperature was best explained with the exponential model fitted to data (Fig. 3.8).

Q_{10} effects on dependency of temperature was relatively stable with only a minor increase from 1.38 for the 13–17 °C range to 1.53 for the 17–21°C range, or for the entire range from 13 to 21°C the Q_{10} coefficient was 1.46.

3.3.2.1.1 U_{crit} and recovery after exhaustive exercise

Critical swimming speed, U_{crit}, exhibited significant changes as the function of acclimated temperatures ($F_{2,\ 34}$ = 51.541, p < 0.0001, Fig. 3.10). Investigation into temperature groups with the multiple comparison Holm-Sidak method showed evidence that the significant increase occurred between 13 and 17°C (t = 8.875, p < 0.0010) and 13 and 21 °C (t = 8.256, p < 0.0010); however, U_{crit} did not change when data from fish acclimated to 17 and 21°C were compared (Fig. 3.10, orange 2nd polynomial order curve and orange symbols).

Q_{10} coefficient for snapper U_{crit} for the temperature array from 13 to 17°C was 2.01, but virtually no dependence on temperature was observed for range from 17 to 21°C with a Q_{10} of 0.97, which brought the overall temperature extent, from 13 to 21°C, Q_{10} to 1.40.

Recovery time after exhaustive exercise, according to one-way ANOVA on reciprocally transformed data (1 data^{-1}), did not differ for temperature groups although a possibility of a trend in the form of a steady reduction in recovery time with rise in temperature may be suggested (Fig. 3.9). Moreover, temperature effects on recovery time can be best explained as the power function decay as illustrated in Fig. 3.9.

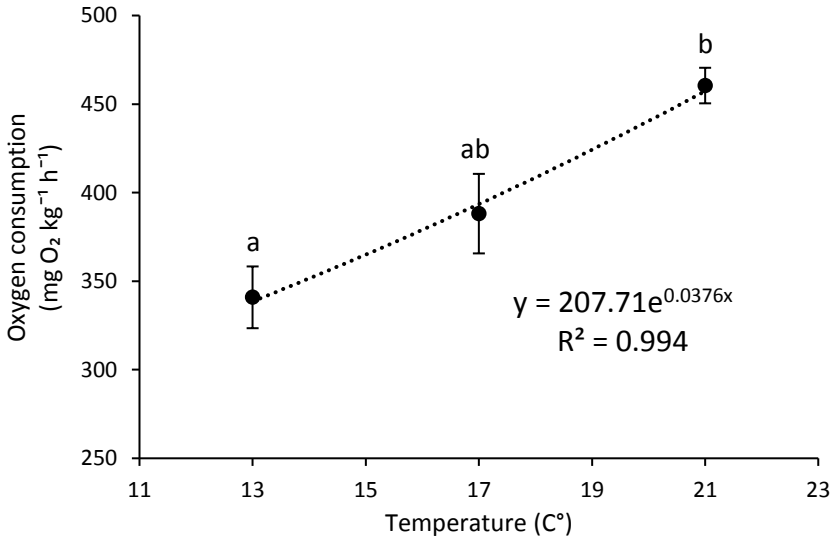

Figure 3.8. Mean mass-specific maximum oxygen consumption (maximum metabolic rate, MMR, mg O_2 kg^{-1} h^{-1}) of snapper as a function of experimental temperature (13, 17 and 21°C). A curve explaining mean values (black symbols) represents the exponential model.

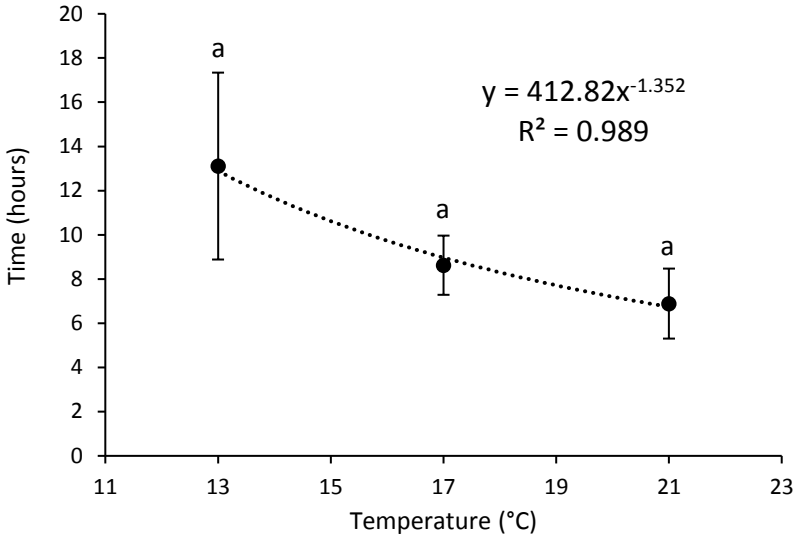

Figure 3.9. Mean recovery times after exhaustive exercise of snapper as a function of experimental temperature (13, 17 and 21°C). A curve explaining mean values (black symbols) represents the power model.

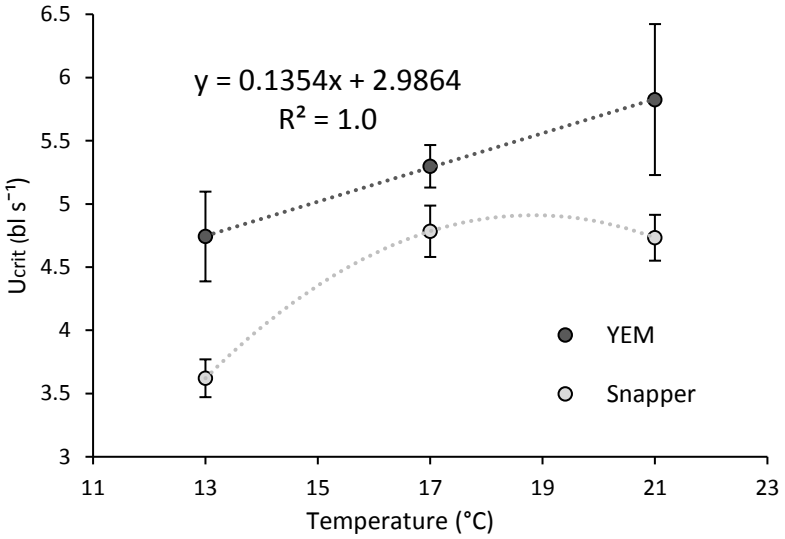

Figure 3.10. Mean critical swimming speeds (U_{crit}) of snapper (light grey symbols with 2nd polynomial curve) and YEM (dark grey symbols and line with associated linear equation and R^2) as a function of experimental temperatures (13, 17 and 21°C).

3.3.2.2 YEM MMR

Temperature had a significant exponential effect on YEM MMR ($F_{2, 22}$ = 12.651, p = 0.0010, Fig. 3.11). Following the ANOVA test, the Holm-Sidak post-hock method identified differences amongst MMR generated for all acclimated temperatures (Table 3.2).

Similarly, to snapper, Q_{10} effect for MMR data showed stability amongst experimental temperatures, hence the Q_{10} for the temperature between 13 and 17°C was 1.49 and a minor increase to 1.54 for the range between 17 and 21°C was observed, making the overall Q_{10} effect for the range from 13 to 21°C, 1.51.

3.3.2.2.1 U_{crit} and recovery after exhaustive exercise

U_{crit} was a YEM parameter that was linearly influenced by temperature (Fig. 3.10). Testing for differences amongst groups revealed that the effect of temperature was significant ($F_{2, 17}$ = 7.226, p = 0.0061). The temperature combination with a significant difference was 13 and 21°C (t = 3.671, p = 0.0072) and 13 and 17°C (t = 2.525, p = 0.0433).

Q_{10} effect on YEM U_{crit} was fairly balanced for the experimental temperatures, it was 1.32 between 13 and 17°C and 1.27 between 17 and 21°C, which made the overall Q_{10} coefficient of 1.29 for the 13 to 21°C range.

The recovery period after exhaustive exercise appeared to be linearly affected by temperature (Fig. 3.12); however, no significant difference amongst the three temperature acclimated groups was observed.

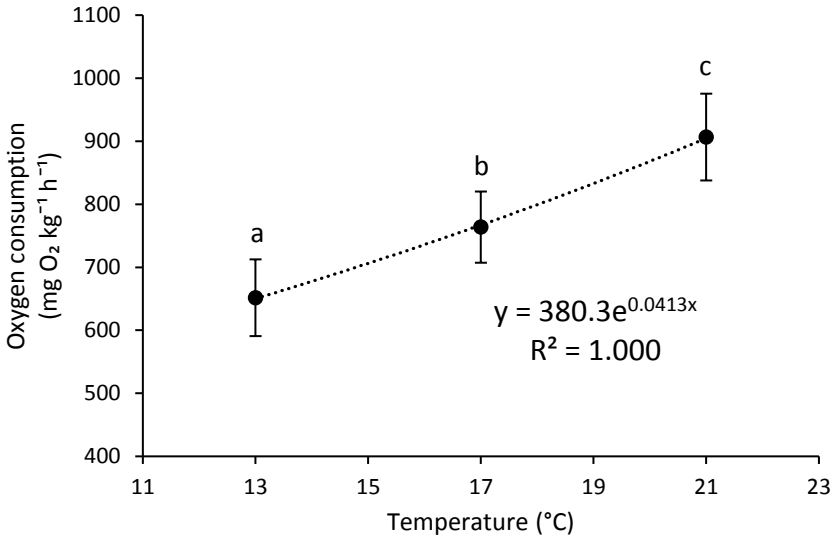

Figure 3.11. Mean mass-specific maximum oxygen consumption (maximum metabolic rate, MMR, mg O_2 kg^{-1} h^{-1}) of YEM as a function of experimental temperature (13, 17 and 21°C). A curve explaining mean values (black symbols) represents the exponential model.

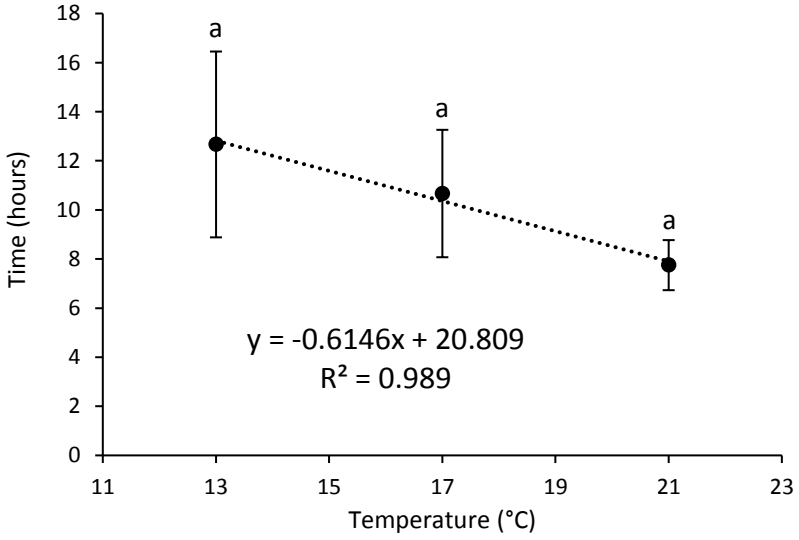

Figure 3.12. Mean recovery times after exhaustive exercise of YEM as a function of experimental temperature (13, 17 and 21°C). A curve explaining mean values (black symbols) represents the linear model.

3.3.2.3 MMR Snapper vs. YEM

MMR between the two species was markedly different, which is evident when data were graphically illustrated (Fig. 3.13) and statistically elaborated (i.e. two-way ANOVA on $\log_{10}$ transformed data ($F_{1, 48}$ = 365.658, $p < 0.0001$). All three-temperature corresponding MMR $\log_{10}$ mean values of the two species differed (Table 3.2). However, since there was no significant interaction term it was suggested that increased temperature had the same effect on both species despite MMR data being best explained by exponential and linear model for snapper and YEM respectively (Fig. 3.13). Metabolic recovery after exhaustive exercise, in contrast, did not differ between the two species for any experimental temperatures (Fig. 3.14).

U_{crit} between snapper and YEM for the array of experimental temperatures was compared when its relative form (i.e. expressed in body length second^{-1}) alongside covariate *length* was analysed with the general linear model ANCOVA (Fig. 3.10). The effect of factor *species* was observed ($F_{1, 46}$ = 68.631, $p <$ 0.0001) with all three U_{crit} mean values at their acclimated temperatures being higher for YEM than snapper (Tukey HSD post-hoc for 13°C $p < 0.0001$; 17°C $p = 0.0010$ and 21°C $p = 0.0190$).
Q_{10} effects on MMR of test species, as mentioned earlier, were fairly similar. This was also true for the standardised data (i.e. 1.38, 1.67 and 1.50, 1.48 for 13–17 and 17–21°C temperature ranges for snapper and YEM respectively). However, Q_{10} effects on U_{crit} showed a different trend where for snapper Q_{10} dropped for a half of its value (i.e. from 2.01 to 0.97) between two temperature blocks (i.e. 13–17 and 17–21°C) while for YEM, Q_{10} remained virtually unchanged (i.e. 1.32 and 1.27) for the same thermal ranges.

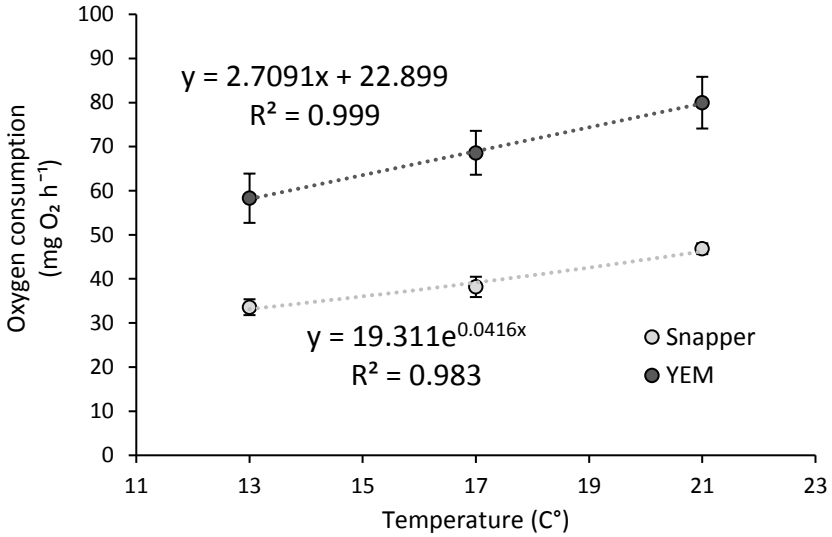

Figure 3.13. Mean values of maximum oxygen consumption (MMR, mg O_2 h^{-1}) standardised to 100 g of snapper (light grey curve and symbols) and YEM (dark grey line and symbols) as a function of experimental temperatures (13, 17 and 21°C). A line and a curve connecting mean values represent the models of best fit (i.e. linear and exponential model for YEM and snapper respectively) with associated equations and R^2.

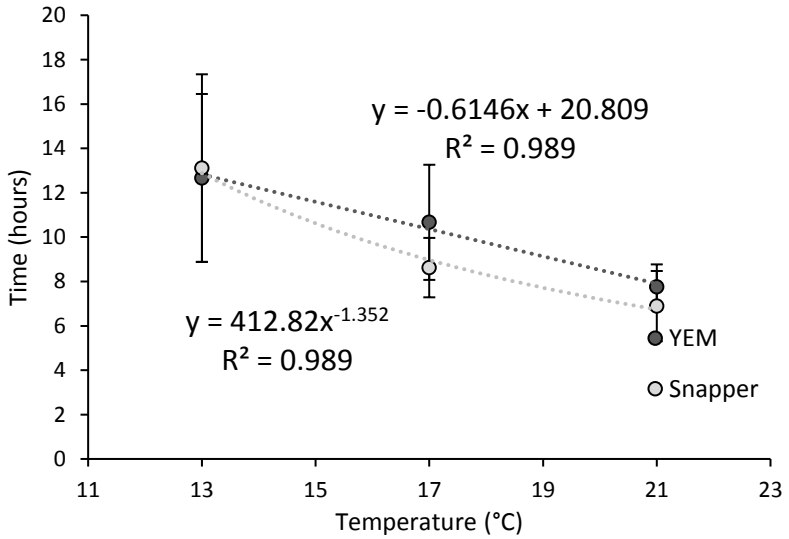

Figure 3.14. Mean recovery times after exhaustive exercise for snapper (light grey curve and symbols) and YEM (dark grey line and symbols) as a function of experimental temperatures (13, 17 and 21°C). Curves connecting the mean values represent the model of best fit (i.e. power model for snapper and linear for YEM) with associated equations and R^2.

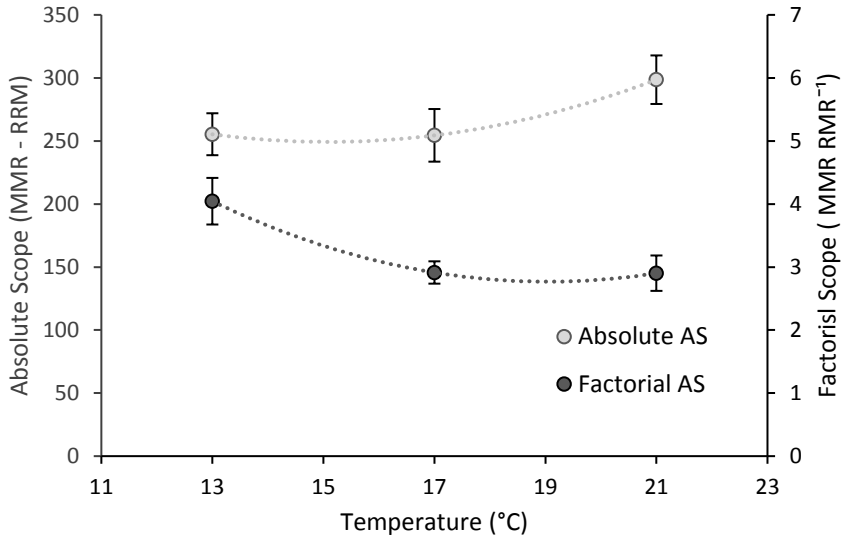

Figure 3.15. Snapper mean absolute (light grey curve and symbols) and factorial aerobic scope (AS) values (dark grey curve and symbols) as a function of acclimated temperatures. Curves are 2nd polynomial function fitted to the data.

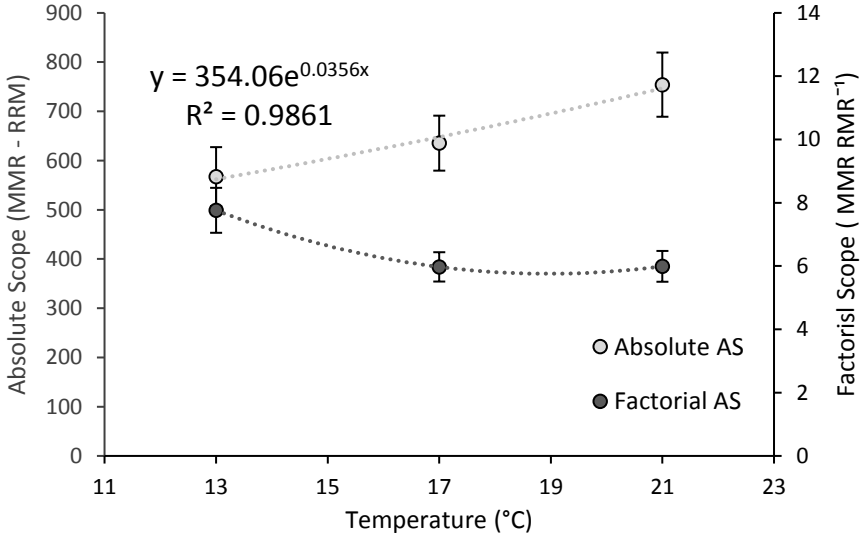

Figure 3.16. YEM mean absolute (light grey symbols and curve with associated exponential equation and R^2) and factorial aerobic scope (AS) values (dark grey symbols with 2nd polynomial curve) as a function of acclimated temperatures.

3.3.3 Aerobic scope (AS)

3.3.3.1 Snapper AS

AS was calculated as absolute (i.e. MMR – RMR, AAS) and factorial (i.e. MMR RMR^{-1}, FAS) metabolic scope. AAS for fish acclimated to different study temperatures differed ($F_{2, 25}$ = 6.173, p = 0.0072, Fig. 3.15) and the post-hoc test revealed the difference between the two sets of temperatures: 13 and 21, and 17 and 21°C (Table 3.2). In other words, AAS was unchanged for 13 and 17°C but it significantly increased at 21°C. However, a somewhat opposite pattern emerged for factorial scope (Fig. 3.15, $F_{2, 25}$ = 17.260, p < 0.0001,). Factorial AAS was the highest at 13°C and differed to the scope calculated for 17 and 21°C which in turn were statistically the same (Table 3.2).

3.3.3.2 YEM AS

Although mean YEM AAS significantly increased with increased experimental temperatures ($F_{2, 22}$ = 7.511, p = 0.0043, Fig. 3.16), the difference between 13 and 17°C was not detected. This fact enabled the exponential model to be the best matrix to explain YEM absolute AS data as a function of temperature (Fig. 3.16). Factorial scope, showed the same behavioural pattern, in respect to temperature as snapper, with an overall significant effect ($F_{2, 22}$ = 10.644, p < 0.0010, Fig. 3.16) and differences observed between means for 13°C with both higher acclimated temperatures (Table 3.2).

3.3.3.3 Snapper vs. YEM

Both types of AS, AAS and FAS, differed between snapper and YEM ($F_{1, 48}$ = 344.106, p < 0.0001, and $F_{1, 48}$ = 355.845, p < 0.0001 for absolute and factorial metabolic scopes respectively, Fig. 3.17A, B) at all acclimated temperatures (Table 3.2). When differences between AASs were tested, two-way ANOVA further revealed a significant interaction between factors *temperature* and *species* ($F_{2, 48}$ = 3.421, p = 0.0420). This signified that temperature had different effects on absolute AS between the two species, where YEM scope increased significantly more with the rise of temperature in comparison with the dynamics observed for snapper (Fig. 3.17A). The way temperature shaped the behaviour of factorial scope appeared to be similar between the two species as there were no interactions between levels of factors *temperature* and *species* observed (Fig. 3.17B). Overall, both YEM scopes were about two times greater for all experimental temperatures. More specifically, YEM absolute scope at 13°C was 2.03 times higher than snapper AAS. The multiplier further increased for the scope of fish acclimated at 17 °C to

2.30, which decreased to 2.23 for 21°C. At the same time factorial scope was again higher for YEM, with the similar multiplier of 2.04 at 13°C, which further increased at 17 °C (i.e. 2.17) and 21°C (i.e. 2.23).

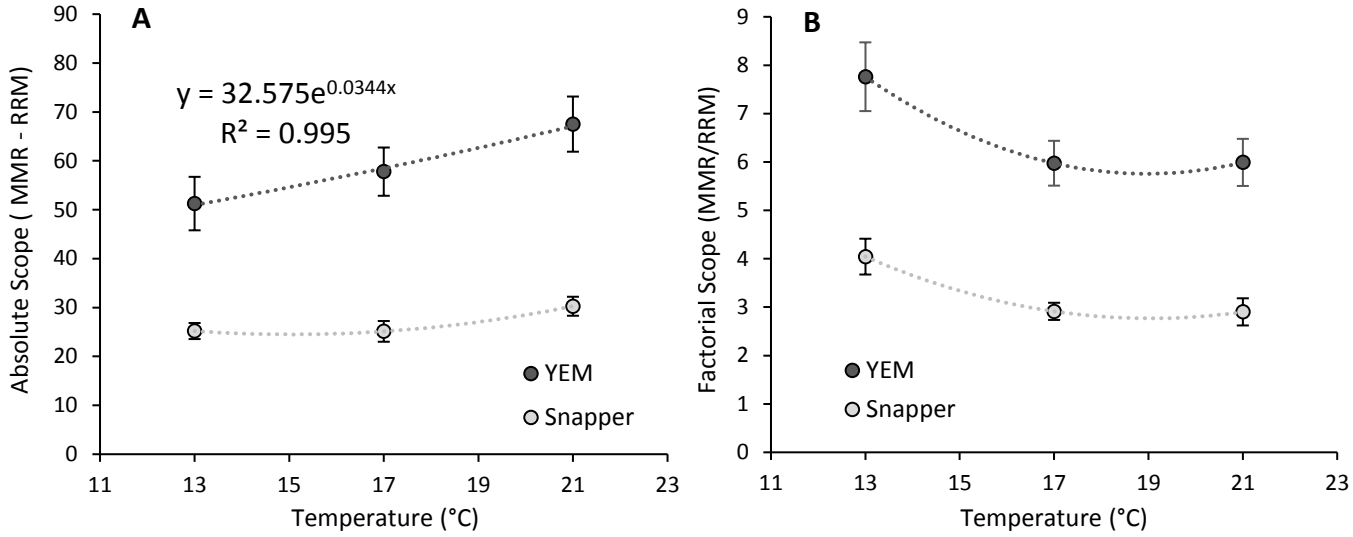

Figure 3.17. A – Mean absolute aerobic scope (AS) of snapper (light grey symbols and curve) and YEM values (dark grey symbols and curve with associated exponential equation and R^2) derived from data standardised to common mass (100 g) as a function of acclimated temperatures. B – Factorial AS of snapper (light grey symbols and curve) and YEM (dark grey symbols and curve) as a function of acclimated temperatures. Curves without associated equation and R^2 are 2nd polynomial function fitted to the data.

Table 3.2. Summary of: **A** – descriptive statistics (mean and standard deviation) of routine (RMR) and maximum (MMR) metabolic rates and absolute (AAS) and factorial (FAS) aerobic metabolic scope of snapper (SN) and yellow-eyed mullet (YEM) for three experimental temperatures (13, 17 and 21°C); **B** – descriptive statistics of the same parameters as in **A** after allometric scaling to the common mass of 100 g; **C** – test statistics with associated p-values for comparison of temperature groups between species of the same parameters as in **B**; **D** – test statistics with associated p-values for comparison of temperature groups within species of the same parameters as in **A.**

		RMR			MMR			AAS			FAS		
A	TEMP	13°	17°	21°	13°	17°	21°	13°	17°	21°	13°	17°	21°
SN	MEAN	87.82	131.63	160.67	340.88	388.14	460.44	255.34	254.45	298.59	4.04	2.91	2.89
	SD	11.28	14.44	21.84	26.68	32.41	15.37	25.46	30.12	29.48	0.57	0.26	0.43
YEM	MEAN	84.05	125.28	152.26	651.67	763.67	906.54	567.57	635.47	754.28	7.76	5.97	5.99
	SD	7.09	9.79	15.78	76.06	86.47	99.34	74.69	85.49	94.12	0.89	0.71	0.69
B													
SN	MEAN	8.58	13.08	16.36	33.59	38.19	46.85	25.21	25.12	30.26	4.07	2.93	2.89
	SD	1.16	1.33	2.47	2.75	3.32	1.88	2.51	3.06	2.97	0.58	0.25	0.43
YEM	MEAN	7.07	10.48	12.49	58.31	68.61	79.97	51.26	57.79	67.51	8.31	6.37	6.46
	SD	0.64	0.91	1.21	6.98	7.62	8.46	6.84	10.81	12.46	0.94	0.77	0.76
C													
SN vs. YEM	TEST -STATS	3.53	6.89	4.93	10.39	12.02	10.92	8.28	11.25	12.83	11.66	10.29	10.68
	P-VALUE	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
D	TEMP	13–17°	17–21°	13–21°	13–17°	17–21°	13–21°	13–17°	17–21°	13–21°	13–17°	17–21°	13–21°
SN	TEST -STATS	7.55	5.01	10.13	2.01	2.24	4.18	0.06	3.01	3.04	4.96	0.05	5.16
	P-VALUE	< 0.001	< 0.001	< 0.001	> 0.05	> 0.05	< 0.05	0.952	0.012	0.017	< 0.001	0.962	< 0.001
YEM	TEST -STATS	8.62	5.64	11.93	2.23	3.09	4.96	1.39	2.65	3.75	4.18	0.04	4.04
	P-VALUE	< 0.001	< 0.001	< 0.001	0.037	0.011	< 0.001	0.178	0.031	0.004	0.001	0.966	0.001

3.4 Discussion

3.4.1 Metabolic phenotype observation – temperature effects

Temperature, as expected, influenced the metabolic phenotype of both test species but not in the same way. Routine metabolic rate (RMR) of snapper and YEM increased with temperature in the same fashion; however, snapper resting metabolism was generally higher and the rate of increase with temperature was stronger suggesting different capacities of thermal compensation between the two species. MMR, on the other hand, was markedly higher for YEM at all times. Taken together as an aerobic capacity (i.e. aerobic scope, AS) to supply tissues with universally needed oxygen, different dynamics of absolute AS (AAS) between species due to temperature could be perceived and it was markedly higher for YEM for all acclimation temperatures. The distinctly higher aerobic capacity of YEM may reflect adaptation to their highly active omnivorous lifestyle.

3.4.1.1 Resting metabolic rate

Standard metabolic rate (SMR) as a description of maintenance or resting metabolic activity in active fish is seldom experienced in the natural setting (Chabot et al., 2016a). SMR may only be relevant to fish with a lifestyle that permits longer motionless resting periods between foraging/prey capture events as is the case for some bottom dwelling and ambush/sit and wait predators. Therefore, in many instances routine metabolic rate (RMR) is more likely to be experienced; nevertheless both metrics are an approximation of baseline maintenance energy expenditure or resting metabolic rate (Clarke and Johnston, 1999; Clarke, 2003; Clarke and Fraser, 2004), which is often closely associated with fish activities, growth performance, social dynamics and lifestyle (Metcalfe et al., 1995, 2016; Clarke and Johnston, 1999; McCarthy, 2000; Cutts et al., 2002; Millidine et al., 2009; Killen et al., 2010). In this thesis RMR rather than SMR was chosen to represent snapper and YEM resting metabolic rates. First, both test species exhibit lifestyles where relatively high level of spontaneous activity precludes SMR as a practical representation of their baseline/resting metabolism. Second, when fish in the stage of intensive growth (i.e. juveniles) are used in bioenergetics research it is recommended to report their resting metabolic rates as RMR, due to the effect of growth on metabolic activity (Chabot et al., 2016a). Since both species in this study were in that stage it was sensible to utilise RMR. Third, another recommendation from the same authors states that if fish in a study exhibit intense gonadal growth, resting metabolic activity has to be reported as RMR. This was clearly the case for YEM since during the monthly sampling sessions, when specimens used for tissue and organ sampling were at ~150 mm length, they showed intense gonadal development. Moreover, whenever the study is in a comparative

form utilising two or more species under the same scrutiny further recommendation is to have a uniform approach to all species for any comparisons to be valid. And finally, for the RMR to be acceptable in the specific dynamic action (SDA, cost of digestion and assimilation; see chapter 4) investigation, it should be maintained at the same level before and after SDA response, otherwise SMR is recommended (Chabot at al., 2016c).

That an increase in ambient temperature is related to an increase in resting metabolism is a well-documented biological phenomenon, which according to Gillooly et al. (2001) is due to thermodynamically higher kinetic energy of cellular components at higher temperatures. However, it is not uncommon to observe animals with low and high resting rates in the same thermal habitats (Clarke and Fraser, 2004). Therefore, Clarke and Fraser (2004) proposed that rates would not increase with temperature merely because of thermodynamics, but that resting metabolism is likely determined by a synergistic input of environmental temperature and a trade-off between costs, benefits and ecological lifestyle. Nevertheless, resting metabolic data, when measured within the temperature range that supports growth, can often be explained as an exponential increase (Jobling 1994; Norin and Clark, 2016). Yet, in the present study, an increase in RMR data with temperature for both test species, was best fitted with the natural logarithmic function. Since *C. auratus* of similar size were also utilised as a model in Coxon (2014), where an exponential increase in RMR from 12 to 18 to 24°C was observed, it remains unclear why the different pattern was found in the present study. However, when data from the present study (13–17–21°C) and Coxon (2014) were combined, the exponential character of the relationship became obvious (Appendix 3A). In respect to YEM, generally there is a scarcity of primary literature addressing metabolic rates and their temperature dependence. Nevertheless, Marais (1978) using *Mugil cephalus*, *Liza richardsonii* and *L. dumerili* from South Africa adapted to higher temperatures than exhibited by YEM from Tasman Bay, showed that resting data may fit a logarithmic trajectory in relation to three intermediate acclimation temperatures (18, 23 and 28°C), but when data for 13 and 33°C were added, again exponential effects of temperature were evident. This demonstrates that temperature/RMR relationship should be discussed with care when rates were measured at intermediate temperature range (i.e. not encompassing entire thermal tolerance zone), since in the present case without the aid of the Coxon (2014) data it could be concluded that resting data have tendency to plateau (as shown with the logarithmic function fit to the current study data). That would mean that effects weaken with the temperature increase, while in fact for snapper and possibly for YEM the effects get stronger with temperature rise as shown with an exponential function fitted to the combined data.

In addition, YEM RMR increase in association with temperature occurred at a lower rate than for *C. auratus*. This may suggest that YEM possess physiological systems capable of compensating for effects of temperature more than snapper. Thermal compensation for a given function for two acclimation temperatures commonly diminishes Q_{10} value to between 1.0 and 2.0, where 1.0 indicates perfect

compensation (Farrell, 2016). Therefore, a certain level of thermal compensation was obvious for both test species (i.e. Q_{10} of 1.75 and 1.55 for snapper and YEM respectively) for the temperature increase from 17 to 21°C. However, the ability to compensate was not observed in either species for the lower experimental temperature range (i.e. 17–13°C) indicated with a Q_{10} closer to 3.0, which was still higher for snapper. Interestingly, Coxon (2014) did not observe any levels of thermal compensation even for the thermal bracket of 18 to 24°C but rather a strong dependence on temperature with a Q_{10} of 3.9. Moreover, she reported an extremely high indication of thermoconformity (i.e. metabolic activity suppresses proportionally to the temperature descent) with a Q_{10} of 4.7 for the range 18–12°C. Coxon (2014) proposed that the reason for the unusually high Q_{10} values could be an artefact of utilising RMR over SMR, which may significantly increase with increased spontaneous activity at higher acclimation temperatures and cause oxygen consumption (MO_2) to rise outside of values associated with SMR (Cossins and Bowler, 1987). However, RMR was the metric of choice in the present study, Ibarz (2003) and Ibarz et al. (2007) using a sister species *Sparus aurata* at comparable size showed a Q_{10} of ~2.5 for the temperature range 18–8°C, which was consistent with snapper thermal dependence observed in the current study. In addition, Requena et al. (1997), demonstrated nearly perfect compensation of small *S. aurata* juveniles (~6.5 g) at a higher temperature range (i.e. 20–28°C with a Q_{10} of 1.1), again relatively comparable to current snapper findings. When exposed to low temperatures analogous values of Q_{10} have also been observed in other Sparid species (e.g. *Lithognathus lithognathus* and *Lithognathus mormyrus*, Du Preez et al., 1986), suggesting lack of capacity to acclimate to cold (Ibarz et al., 2010). Similar to findings of YEM, mullet RMR from Marais (1978) also exhibited a tendency for thermal compensation at higher but to conform at lower acclimation temperatures.

A temperature of 12–13°C has been proposed to be a threshold for metabolic activity in *S. aurata* (Ibarz et al., 2010) and it is often a recognised temperature edge associated with a drop in snapper activity, feeding and growth. A detailed comparison of RMR data from Coxon (2014) measured at 12°C, with the data from the current study obtained at 13°C revealed a very clear-cut difference between the two temperatures where markedly higher rates accompanied 13°C (this study) relative to 12°C (Coxon, 2014) (Appendix 3B). This cannot be explained by possible differences in methodologies, fish size, origin (genetic differences) or husbandry procedures due to high similarity of those factors and the same source of experimental subjects (i.e. PFR) for both studies. Furthermore, when the same comparison was conducted for 17°C (present study) and 18°C (Coxon, 2014), virtually no difference in RMR was observed (Appendix 3B). Almost identical patterns were present for MMR also. This finding strongly coincides with the proposed *S. aurata* metabolic temperature verge (Ibarz et al., 2003, 2010) indicating a stepwise transition in metabolic performance with temperature drop from 13 to 12°C rather than gradual adjustments of physiological machinery to approaching detrimental temperatures. This possible rapid metabolic modification may elicit ionic imbalance, alter plasma homeostasis and liver metabolism, impair immune competence and food intake and increased

susceptibilities to infections, which is ultimately related to the onset of the winter syndrome in *S. aurata* (Ibarz et al., 2010). This may also be important and highlight *C. auratus* inability to withstand lower temperatures.

3.4.1.2 Maximum metabolic rate

Similar to resting metabolism, MMR also often increases exponentially with acclimation temperature; however, it may start plateauing at some intermediate thermal levels and soon after T_{optMMR} (optimal temperature for MMR) can be reached. With further temperature increase MMR decreases when negative effects on oxygen supply and its delivery become apparent (Farrell et al., 2009; Pörtner, 2010). However, evidence is emerging showing fish taxa with MMR's continuous increase with temperature so that T_{optMMR} is found near the upper critical thermal limit rather than at some intermediate thermal levels (Norin and Clark, 2016). In this study, in contrast to RMR, MMR exhibited an exponential increase with a rise in acclimation temperature for both test species (apart from YEM standardised data used for direct comparison between the two test species, where increase was linear), which demonstrated no indication of plateauing and thus approaching T_{optMMR}. Therefore, the YEM data suggest that T_{optMMR} should be attained above 21°C or at the very edge of the temperature range experienced in natural habitats (~23°C) or even beyond that level. However experimental verification of this hypothesis would be warranted. In the case of snapper, Coxon (2014) provided insight to that question since MMR in her study increased exponentially with temperature and data similarly showed that there were no signs that T_{optMMR} was near, even at the highest acclimation level (i.e. 24°C), which is already a thermal maximum unlikely to be experienced by the snapper population adapted to the northern South Island annual thermal sea-water oscillation. Therefore, it appears that both species in the present study follow the concept illustrated in Norin and Clark (2016) where MMR continues to increase with temperature likely reaching T_{optMMR} near their upper thermal tolerance boundary.

In terms of Q_{10} effects on MMR, both test species appeared to have similar temperature dependency for the two temperature increases (i.e. 13–17 and 17–21°C) with an average value of 1.5. Overall average for MMR Q_{10} effects was lower than values observed for RMR (i.e. ~2), therefore it appeared that when fish operated at maximum level of oxygen utilisation that they were capable of better thermal compensation than what was possible during rest. However, this may be just an artefact of a limitation of the cardio-respiratory system and/or capacity of tissues and mitochondria to further increase aerobic functioning (Farrell, 2002; Steinhausen et al. 2008). Therefore, it is possible that the limitation masked the effects of temperature to match MMR Q_{10} values with those observed for RMR at the given temperature range. The limitation appears to be more intense at lower temperatures for

both species, however, still more evident for snapper. Similarly, even though higher snapper MMR Q_{10} values were reported in Coxon (2014; 1.9 and 2.1 for 18 – 24 and 12 – 18°C ranges respectively); a comparable drop in thermal sensitivity between RMR and MMR was noted.

The most notable difference between snapper and YEM MMR was in actual values of MO_2, which were markedly higher for YEM at all three acclimation temperatures (i.e. >70%). This is not a surprise since interspecific differences in MMR, due to differences in their lifestyle as a reflection of adaptation to specific environmental conditions and frequency of change in biotic and abiotic variables, may differ by more than 10-fold (Norin and Clark, 2016). This adaptation will have a pronounced impact on the extent of aerobic capacity for activity or aerobic scope. The lowest MMR is usually measured for flat-fish and ambush predators (e.g. *Solea sole*a and *Esox Lucius*) with MMR values less than 200 mg O_2 kg^{-1} h^{-1}, and the highest for salmonids (particularly genus *Oncorhynchus*), yellowtail kingfish (*Seriola lalandi*) and at the very extreme end tropical bluelined wrasse (*Stethojulis bandanensis*), which is capable of MMR of up to 2800 mg O_2 kg^{-1} h^{-1} (Norin and Clark 2016). YEM MMR for some individuals exceeded 1000 mg O_2 kg^{-1} h^{-1} at 21°C, and similar levels of oxygen utilisation were repeatedly observed for golden grey mullet (*Liza aurata*, Milinkovich et al., 2012; Vagner et al., 2014). This places YEM and possibly the entire mullet family amongst the *Oncorhynchus* spp. with a capability of high oxygen uptake and delivery capacity for aerobic functions as required for perpetual foraging swimming mode (mullets and kingfish), or as required for across ocean spawning migration to freshwater environments (salmonids) (Lee et al., 2003; Farrell, 2007; Clark et al., 2011; Eliason et al., 2011). In contrast, snapper is a shoaling, demersal fish and do not require high aerobic performance in everyday life, therefore a smaller aerobic scope and related sustained swimming capacity could be expected when compared with YEM.

3.4.1.2.1 Critical swimming speed (U_{crit})

Sustained swimming is activity resulting from the synergistic input of physiological (i.e. metabolic, muscular and cardio-respiratory functions) and physical (i.e. hydrodynamics) components that all have a propensity to differ with change in temperature, fish size and species (Marsh, 1990; Taylor et al., 2008). Adaptations to different ecological niches through specialised lifestyle and body forms require diverse locomotory abilities. For example, zebrafish (*Danio rerio*) possess an extreme capacity for sustained swimming (i.e. 18 body lengths per second, bls^{-1}), which is clearly related to their relatively small size (~3.60 cm total body length) (Palstra et al., 2010). Chub mackerel (*Scomber japonicus*) and skipjack tuna (*Euthynnus lineatus*) are capable of sustained swimming for prolonged periods of time at high velocities of up to 8 bls^{-1} (Magnuson, 1978; Sepulveda and Dickson, 2000). Similarly, flathead grey mullet *M. cephalus* were observed to employ a cruising speed of 7 bls^{-1} during migration along the mid-Atlantic coast of North America (Peterson, 1976). Whereas species adapted

to the benthos such as flatfish (e.g. North Sea plaice, *Pleuronectes platessa*, 1.1–1.5 bls^{-1}; Priede and Holliday, 1980) or benthopelagic environments like cods (e.g. the Atlantic cod, *Gadus morhua*, 1.25–1.75 bls^{-1}; Martínez et al., 2004) exhibit markedly lower faculties for sustained swimming.

C. auratus could be classified somewhere in the middle of this range as a species that can sustain moderate swimming velocities, which is in accord with their demersal-pelagic lifestyle (Mossman, 2008). This can be verified with an overall U_{crit} average of 4.8 (12, 18, 24°C) and 4.4 (13, 17, 21°C) bls^{-1} observed in Coxon (2014) and the present study respectively. On the other hand mullet, such as YEM appeared to be more agile performers (i.e. overall average of 5.3 bls^{-1} for 13, 17 and 21°C), which was further supported with findings from work conducted on *L. aurata* where a relatively high U_{crit} (i.e. 6.5–7 bls^{-1}) was observed even at lower acclimation temperatures (i.e. 15 and 20°C, Milinkovich et al., 2012 and Vagner et al., 2014 respectively) than in the present study. Therefore, the overall impression about mullet swimming performance indicates that the family Mugllidae can be numbered amongst the top swimmers, possibly based on their high MMR and resulting aerobic scope (Milinkovich et al., 2012 and Vagner et al., 2014, this study).

In the present study snapper swimming performance with respect to temperature was comparable to slightly larger fish used in Coxon (2014) (i.e. ~100 and ~140 g fish used in the present and Coxon, 2014 study respectively). U_{crit} in both studies tended to increase to 17/18°C and it was maintained or slightly dropped at subsequent higher temperatures. Nevertheless, combined data from both studies allowed for assessment of $T_{optUcrit}$ (the optimal temperature at which U_{crit} is maximised) for snapper of 100–140 g, which was estimated to be at 19 – 20°C (i.e. 19.6°C). This estimate was not possible for YEM since there is no comparable study to merge data with as was the case for snapper. Nevertheless, YEM U_{crit} as a function of temperature demonstrated a positive linear relationship, suggesting that their $T_{optUcrit}$ for the size ~30 g should be found beyond 21°C. The pattern portrayed in the thermal performance curve, as seen for snapper (i.e. increase-plateau-decrease), is frequently observed for many species for both acute and acclimation temperature exposures (Brett and Glass, 1973; Taylor et al., 1996; Lee et al., 2003).

$T_{optUcrit}$ is usually linked to temperatures experienced in natural environments and, similar to other performances such as reproductive and somatic growth, is often found in close proximity to the highest temperatures encountered in natural habitats that are still within a sufficient distance (temperature buffer zone) from harmful effects of the upper critical thermal limit (Hammer, 1995; Lee et al., 2003; Payne et al 2016). This notion seems to be aligned with estimated snapper $T_{optUcrit}$ since upper temperatures associated with Nelson Haven (Tasman Bay) can peak around 23°C (according to PFR monitoring station). However, this appears to be only partially consistent with other aspects of snapper performances (see below). Despite that, the thermal performance curves for U_{crit} and aerobic

scope commonly exhibit the same pattern as a function of environmental temperatures (Farrell, 2002).

3.4.1.3 Aerobic scope (AS)

Growth potential and overall fitness levels of fish are often associated with oxygen transport capacity that is performed by coordination within the cardio-respiratory system supplying oxygen for all activities beyond basic homeostatic maintenance (Farrell et al., 2008; Neuheimer et al., 2011). This aerobic capacity is commonly expressed as absolute (AAS) or factorial (proportional) scope (FAS). In the literature there is no clear agreement when the use of one of the other is more appropriate, since in practice often conflicting arguments accompany the same data sets (Clark et al., 2013). This appears true for both test species in this study and particularly for snapper. Moreover, when snapper and YEM AS are compared as FAS, the same pattern as a function of temperature is observed, but only when AS was presented as absolute (AAS) the different responses to temperature increase between two species become notable. Nevertheless, factorial differences between YEM and snapper AS seem to be preserved between the two metrics (i.e. YEM AS is more than two times higher for any given experimental temperatures). In addition, Clark et al., (2013) argued FAS as a ratio does not convey any information about the real aerobic capacity when in fact it is about the amount of energy required for a particular action, therefore they recommended systematic utilisation of AAS. Besides, if FAS is to be discussed the findings should be interpreted in an ecological context and use of the metric justified (Clark et al., 2013). Nevertheless, Killen et al., (2007) stated that FAS is more suitable for comparing the aerobic capacity of animals across large size ranges. To fulfil the recommendation from Clark et al. (2013) in this thesis both metrics were presented.

Similar to previously described comparisons of RMR/MMR, snapper AAS data when compared with Coxon (2014), further indicated a potential significance at the 12/13°C threshold for metabolic activity for the species. In Coxon (2014) a 50% increase in AAS can be noted between 12 and 18°C whereas virtually no difference in AAS between 13 and 17°C was observed in the present study. In addition, a ~20% AAS increase accompanied a change of temperature from 17 to 21°C in the current study compared with only 6% between 18 and 24°C in Coxon (2014). The minute AAS increase between 18 and 24°C in her study was accounted for by the effect of plateauing and approach to the T_{optAS} (optimum temperature for AS) that may be reached in close proximity beyond 24°C. This value is within the range that is not commonly experienced by snapper in the Tasman/Golden Bay even during the warmest season, as previously stated.

AAS response to increased temperature in many instances can be portrayed as a bell-shaped curve (Pörtner and Farrell, 2008; Pörtner, 2010). However, as mentioned earlier, there are many examples showing that MMR does not always reach its maximum at some intermediate temperatures and thereafter decreases when approaching the upper critical thermal limit (Norin and Clarke, 2016). Therefore, the AAS curve is more likely to have a distinctly left skewed shape, such as has been observed for European seabass *Dicentrarchus labrax*, *O. nerka*, Atlantic halibut *Hippoglossus hippoglossus*, *G. morhua* and many others (Lee et al., 2003; Claireaux et al., 2006; Grans et al., 2014; Tirsgaard et al., 2015).

OCLTT (oxygen- and capacity-limited thermal tolerance) hypothesis proposes that maximum AS allows for maximum performance or in other words that a fish's capacity to deliver oxygen to tissues becomes limited at temperature extremes (Pörtner, 2001, 2010; Pörtner and Farrell, 2008). The relevance of the hypothesis has been demonstrated in many instances in laboratory and field studies. For illustration, it has been reported that OCLTT assisted in setting southern distribution boundaries in European eelpout (Pörtner and Knust, 2007); and also, OCLTT was estimated to likely be a major limitation during spawning migration of Pacific salmon (Farrell et al., 2008). Nevertheless, in opposition to the OCLITT hypothesis, evidence showing species with maximum potential for different activities achieved at temperatures different to those that support T_{optAS} is steadily accumulating (Clark et al., 2013). For example, killifish (*Fundulus heteroclitus*; 'northern population') exhibited negative specific growth rate (SGR) when acclimated to temperatures representing T_{opAS} (25–30°C) suggesting mechanisms other than oxygen delivery potential to limit growth, while at the same time the gonadosomatic index (proxy for reproductive output) was highest at acclimation temperatures of 10–15°C (Healy and Schulte, 2012). Similarly, Pacific pink salmon *Oncorhynchus gorbuscha* have T_{optAS} at 21°C (Clark at al., 2011) whereas optimal temperature for successful spawning is achieved at temperatures below 14°C. Therefore, an alternative perspective, *multiple performances – multiple optima* was suggested stemming from numerous examples in the literatures mismatching optimal temperatures for aerobic activities (Clark et al., 2013). This viewpoint appears to better describe findings from the present study since both test species seemed to have different thermal optima for some tested aerobic activities. Snapper T_{optAS} is placed above 24°C, around which level T_{opt} for MMR and for SGR could be found; at least T_{optMMR} is beyond 21 and 24°C in regards to this study and Coxon (2014) respectively; and SGR was found to continuously increase with temperature, thus it is safe to suggest that T_{optSGR} (temperature at which SGR is maximised) is above the highest experienced temperatures of 22.6°C. But as explained earlier snapper peak swimming capacity was estimated at ~20°C. In contrast, YEM T_{optAS}, T_{optMMR} and also $T_{optUcrit}$ are clearly beyond 21°C (demonstrated by linear/exponential increase still at 21°C without any indication of plateauing); however, SGR was observed to be maximised at 17/18°C. Therefore, it appears that all described aerobic functions (except for U_{crit} in snapper and SGR in YEM) including AS may reach their maximum beyond the temperature range naturally experienced by the test species. Therefore, the ecological relevance of

T_{optAS} may have weaker predictable power to demonstrate the optimal temperature for organisms than previously thought if AS continues to increase throughout ecologically relevant temperature ranges (Claireaux et al., 2000; Clark et al., 2011; 2013). Accordingly, it seems plausible that interdependence/interconnection between different aerobic functions with different thermal optima may direct selection of the thermal preference in support of fish survival and fitness (Clarke et al., 2013; Farrell, 2013).

3.4.2 Concluding remarks

Resting metabolism encompasses ATP requirements of many vital body processes and therefore signifies the cost of living a specific lifestyle within a specific thermal window (Clarke, 1993). MMR represents the upper limit of the rate that oxygen can be conveyed from the surroundings to tissue mitochondria for all major ecologically and biologically important activities (Norin and Clark, 2013). However, it is about the dynamics between the two that will set the boundaries for aerobic performances and, as it has been elaborated here, temperature is one of the factors that govern them. But the adaptation to a particular lifestyle may also shape the dynamics as seen in differences of the metabolic phenotype of snapper and YEM that may often (at least during particular times in their life history) share the same thermal coastal environments. However, there are many other modulators of AS amongst which the cost of digestion is often listed as an important constituent of fish energy budget and it can significantly affect the aerobic performance of fish (Fu et al., 2009).

Every organism aims not only to survive (this is the minimum requirement) but to thrive by maintaining a good reproductive and health fitness, and to increase scope for activity is one way to achieve this goal (Claireaux and Lefrançois, 2007). To minimise the probability of mortality, greater AS may offer a better metabolic security margin. For example, the cost of digestion and assimilation (i.e. SDA) can in some instances markedly shrink AS and any movement into the pool of aerobic capacity that is still available during digestion may not be sufficient to provide enough energy, for instance to evade a predator (Priede, 1977; Claireaux and Lefrançois, 2007). This scenario is certainly feasible for benthic ambush predators and fish like Atlantic cod where exhaustion of over 90% of AS can be accounted for by SDA (Soofiani and Hawkins, 1982; Claireaux et al., 2000).

Thus, feeding and digestion cost may have significant proportional input to overall energy budget and consequently it can affect growth and survival potential by diminishing AS and consequently reduce the metabolic security margin. Therefore, in the light of exploration of growth capacity, determination of those costs (i.e. SDA) may be a rewarding area of investigation to aid better understanding of actual growth capacity of potential aquaculture/stock enhancement candidates.

CHAPTER 4

Effects of temperature and meal size on specific dynamic action of snapper and YEM

4.1 Introduction

4.1.1 Specific Dynamic Action (SDA) – general overview

Fish like any other animal strive to maintain a positive energy balance that brings essential ecological benefits in terms of growth and reproduction (Nelson and Chabot, 2011). Those benefits can only be fuelled by excess energy remaining after a meal has passed through the body – the process known as energy budget (Secor, 2009). There are several ways of expressing the energy budget in fish but most authors would agree that this is a balance between ingested meal energy (*IE*) on one side:

$$IE = M\ (RMR + AMR + SDA) + FN + SG + FS$$

and on the other is energy used for metabolic activity (*M*, which consists of resting/routine metabolic rate [RMR], active metabolic rate [AMR] and specific dynamic action [SDA]); energy lost through faeces and nitrogenous waste excretion (*FN*), energy built into the fish body through somatic and gonadal growth (*SG*) and energy incorporated into fat stores (*FS*) (Congdon et al., 1982; Cui et al., 1994; Pirozzi and Booth, 2009; Lucas and Watson, 2014).

Specific dynamic action (SDA) is a broadly accepted term that describes an increase in metabolic rate after ingestion of a meal due to digestion, absorption and assimilation of a meal (McCue, 2006; Secor, 2009). The importance of SDA in the overall energy budget of a fish is substantial since it can be responsible for 25–50% of total metabolic costs and can correspond to 10–20% of energy contained in a meal (Xie et al., 1997; Owen, 2001; Jobling, 1983). In some extreme cases almost the entire extent of aerobic metabolic scope (AS) can be occupied by SDA response as has been observed for Atlantic cod (*Gadus morhua*, Soofiani and Hawkins ,1982; Claireaux et al., 2000). Furthermore, a maximum increase in metabolic rate due to SDA can vary from 25% in humans, to 140% in fish, and

to a staggering 700% in snakes (Secor, 2009). SDA response has been described in almost all animal taxa and with its first documented observation dating nearly 200 years ago it has been the centre of numerous physiological studies related to digestion, metabolism and growth (McCue, 2006).

The term Specific Dynamic Action originated in the German language where "specific dynamic effects" as described by Max Rubner in the late 19th century while working on the postprandial response in dogs, has been erroneously translated (Whitney and Rolfes, 1996). Nevertheless, nowadays, the term SDA has been generally acknowledged to represent the phenomenon even though it does not clearly depict the process related to feeding and metabolism (Secor, 2009).

Throughout the history of SDA determination many hypotheses have been proposed to explain the phenomenon and in general terms they can be summed up into three categories – preabsorptive, absorptive, and postabsorptive (McCue, 2006). The preabsorptive or mechanical category tries to explain SDA as the cost of food capture, handling and swallowing, heating of ingested meal (in the case of endotherms), enzyme and acid secretion, intestinal motility, protein catabolism and blood pH regulation (Houlihan, 1991; Wilson and Culik, 1991; Owen, 2001; Secor, 2003). The absorptive category describes SDA as energy cost due to intestinal absorption and nutrient transport (Secor, 2003; McCue et al., 2005). The postabsorptive approach explains SDA as a cost related to protein anabolism, ketogenesis, glycogenesis, urea production and general costs of growth (Kalarani and Davies, 1994; Whiteley et al., 2001; McCue et al., 2005). Nevertheless, the mechanical costs of processing food were found to be minute and, in many cases, insignificant (i.e. 1–3 % of total energy used during SDA) (Jobling, 1993; Peck, 1998). On the other hand, it is considered that up to 80% of SDA ensues from postabsorptive metabolic activity (Brown and Cameron, 1991; Willmer et al., 2004; Seth et al., 2010), of which up to 50% could be related to protein synthesis itself (Aoyagi et al., 1988; Lyndon et al., 1992). Generally, in fish the SDA response appears to start with a rapid increase in metabolic rate with the highest point reached within 3–12 hours followed by steady and slow return to pre-feeding resting rates (Secor, 2009).

To characterise postprandial energy expenditure several metrics have been proposed, each with the purpose of identifying changes in various physiological processes associated with SDA response (McCue, 2006). Of particular interest are: SDA duration (time from ingestion of a meal to the moment when metabolic response has returned to pre-feeding levels); magnitude of SDA cost (integrated values between baseline reference line [e.g. RMR] and a curve above it that represents SDA response); SDA peak (the highest metabolic response due to SDA); time to peak (time elapsed from meal ingestion to the moment when the peak is reached); the SDA factorial scope (ratio between maximal postprandial metabolic rate at the peak and baseline level); absolute increase in metabolic rate or SDA amplitude (the difference between SDA peak and the baseline level); SDA coefficient (percentage ratio of energy expenditure due to SDA and energy enclosed in a meal) (Jobling, 1981a; Secor and Faulkner,

2002); and limitation of AS for activity (percentage of AS reduction during the SDA peak) (Jordan and Steffensen, 2007). There are also many factors that can significantly affect described parameters. The most prominent for ectothermic organisms are ambient temperature, characteristics of a meal: quantity (i.e. meal size/ration) and quality (composition, type), body size (allometric effects), and O_2 saturation (Boyce and Clarke, 1997; Guinea and Femandez, 1997; Herbing and White, 2002; Peck et al., 2005; Jordan and Steffensen, 2007; Pirozzi and Booth, 2009; Secor, 2009; Khan et al., 2015).

When SDA response is investigated with the purpose of improving understanding of energy budgets as well as growth capacities and limitations of species from temperate environments in association with their potential for commercial exploitation, the two SDA modulators that should first be addressed are ambient temperature and meal size. Temperature, is important due to its pervasive effects on all aspects of fish biology and especially in environments where thermal seasonal oscillation is a dominant feature (Jobling, 1997); meal size is a crucially important aspect for investigation of maximising growth efficiency in relation to potential economic exploitation (Khan et al., 2015).

4.1.2 Effects of temperature on SDA

The effects of a rise in ambient temperature on SDA response in fish may vary from an increase in SDA cost, such as in *G. morhua, Silurus meridionalis*, and *Sparus aurata* (Hailey and Davies, 1987; Guinea and Fernandez, 1997; Luo and Xie, 2008), no effects as in *D. labrax, Pleuronectes platessa*, and *Odontobutis obscura* (Jobling and Davies, 1980; Machida, 1981; Peres and Oliva-Teles, 2001), to a decrease in SDA magnitude as has been shown for common minnow, *Phoxinus phoxinus* (Cui and Wootton, 1988). However, the SDA peak normally increases with elevated temperature, which may be due to two occurrences – an increase in resting metabolic rates, and an increase in SDA amplitude (Secor, 2009; Pang et al., 2011; Frisk et al., 2013; Fig. 4.1). This scenario may have strong implications from a global warming perspective since increased temperatures may pose metabolic challenges in relation to quality of feeding and digestion and ultimately growth and reproduction since overall aerobic capacity (i.e. AS) may be reduced (Sandblom et al., 2014). Moreover, an increase in temperature would ultimately shorten the duration of SDA response (Machida 1981; Wang et al. 2002; Secor et al. 2007; Fig. 4.1). In addition, post-feeding metabolic response exhibits its peak sooner and the curve depicting the SDA response is slimmer and taller at higher compared to lower temperatures (Wang et al. 2002; Secor et al., 2007; Fig. 4.1). Since peak SDA appears to simultaneously increase with resting metabolism it is expected that the SDA factorial scope will remain unchanged as temperature increases (Sacor, 2009). There are many studies to support this notion (e.g. Machida, 1981; Soofiani and Hawkins, 1982; Peck et al., 2003); however, generalisation is not permissible due to the body of

evidence demonstrating both an increase and decrease of scope with an increase in temperature (e.g. Hamada and Ida, 1973; Robertson et al., 2002).

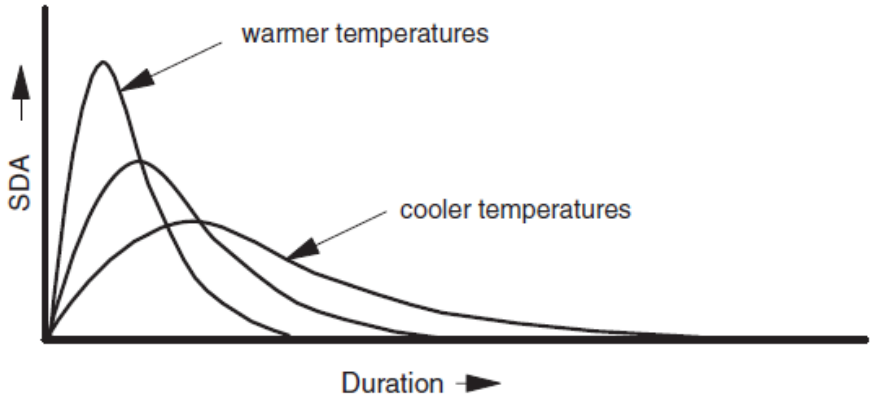

Figure 4.1. Schematic illustration of temperature effects on SDA response in ectotherms. Image sourced from McCue (2006).

4.1.3 Effects of meal size on SDA

Many authors have shown a significant impact of meal size on SDA which can be manifested as an increase in SDA peak, SDA duration and magnitude in linear fashion with increased meal size (Ross et al., 1992; Fu et al., 2005). The profile of this response can be attributed to an increase in time and energy input that is required to digest and incorporate a larger meal into body mass (Secor, 2009). Conversely, SDA peak could also plateau with gradually increased meal sizes (Jobling and Davies, 1980; Secor and Boehm, 2006) and thus reach a maximum SDA level that is constrained by the limiting oxidative capacity of the digestive organs (Jobling and Davies, 1980). In contrast to SDA peak, duration and cost which generally increase with meal size, SDA coefficient may remain unaffected (Beamish, 1974; Jobling and Davies, 1980; Tandler and Beamish, 1980; Paakkonen and Lyytikainen, 2000; Fu et al., 2005), increase (Soofiani and Hawkins, 1982; Chakraborty et al., 1992) or decrease (Boyce and Clarke, 1997; Guinea and Fernandez, 1997). This led Fu et al. (2005, 2006) to propose that the SDA response to ration size is possibly linked to the species dependent feeding strategy as well as the capacity of the cardio-respiratory system.

Observing SDA response through ecological lenses, Alsop and Wood (1997) suggested that fish may not be capable of directing enough energy to maximise the performance of other aerobic activities (i.e.

locomotion) while they are engaged in the digestion of a meal. This concept stemmed from observation of impaired swimming capacity of postprandial rainbow trout (*Oncorhynchus mykiss*) relative to fasted fish (Alsop and Wood, 1997). This paradigm suggests that fish have to partition energy resources between the demands for SDA and other aerobic activities, whereby meal size may act as a trade-off modulator simultaneously causing SDA metabolism to increase and capacity of aerobic scope for other activities to decrease (Owen, 2001; Fig. 4.2)

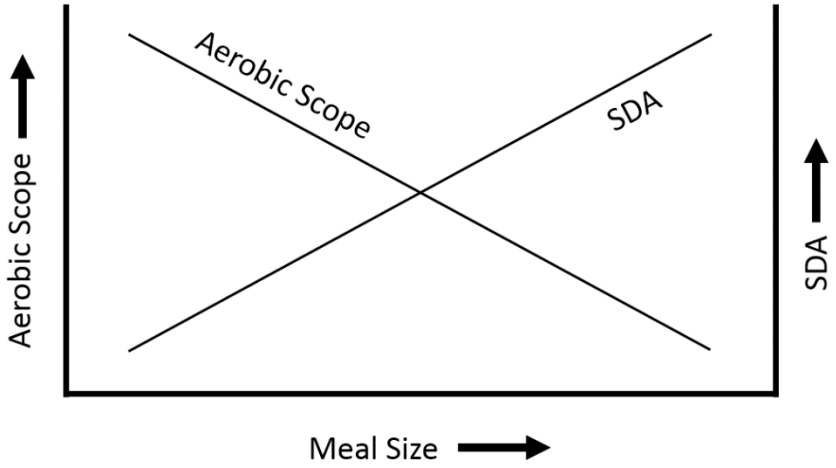

Figure 4.2. Schematic illustration of possible trade-off between aerobic scope for activity and metabolic increase due to SDA. Image sourced from Owen (2001).

4.1.4 Objectives of the chapter

SDA is an inevitable cost to organisms. However, with clear benefits to growth in terms of nutrient assimilation and protein synthesis, SDA cannot be viewed as a pure metabolic loss (Mallekh and Lagardere, 2002) but rather as the cost of growth (Jobling, 1981b). Having this in mind, when SDA is inspected from an economic vantage point, in an attempt to enhance the efficiency of production, trying to minimise SDA cost may not be the most practical approach as this may cause a decline in growth rates (Lyndon et al., 1992). Thus, the main purpose of studies on fish taxa in regards to aquaculture, commercial or recreational fishing has been to detect optimal conditions that would minimise certain aspects of SDA response (i.e. duration, SDA coefficient), which may allow higher amounts of energy extracted from a meal to be partitioned into somatic or gonadal growth (Chakraborty et al. 1992; Peres and Oliva-Teles 2001; Fu and Xie 2004).

The objectives of the chapter can be outlined as the characterisation of a set of parameters related to metabolic alteration that follow ingestion of meal at three acclimation temperatures 13, 17 and 21°C, spanning the range commonly experienced in the natural environment, at which snapper would feed voluntarily in the respirometer. The work used a predetermined ration of 0.5 % BM (body mass) to examine temperature effects on SDA, and four rations (0.5, 1, 2 and 3% BM) at the set temperature of 17 °C to assess the effects of meal size on SDA response. Ultimately, the findings of the chapter are aimed to be linked with growth performance (chapter 2) and metabolic activity (chapter 3) of test species which is elaborated in chapter 7.

This thesis, apart from seeking to describe biological growth properties of two different coastal and temperate finfish species in light of physiological and ecological context, also aims to contribute to an evaluation of further commercial and recreational exploitation capacities of test species, for which it is believed that SDA profile characterisation may be beneficial. This work appears to be the first conducted on juvenile snapper *C. auratus* and yellow-eyed mullet *A. forsteri* to date.

4.2. Materials and methods

All general methodology including acquisition of experimental subjects, husbandry, acclimation to testing temperatures, respirometry and Animal Ethics consent were as described in chapter 3 under Materials and Methods (3.2). This section on Materials and Methods will cover only the specifics of the SDA work, which includes experimental design and protocols, determination of SDA parameters and statistical analyses.

4.2.1 Setting up Levels of SDA treatments for Snapper and YEM

4.2.1.1 Effects of temperature on SDA

Snapper and YEM were scheduled to be fed with a set ration of 0.5% body mass (BM) at the three test temperatures of 13, 17 and 21°C. This was successfully executed for snapper only. YEM, using the mirror (see 3.2.3.2.2) and feeding tube (see 4.2.2.2) set up in the respirometer, were successfully fed at 21°C, and enough animals were sufficiently fed for SDA determinates to be calculated at 17°C.

However, YEM completely refused to take a meal at 13°C. Therefore, no SDA data were obtained at this temperature for YEM.

4.2.1.2 Effects of ration size on SDA

The second objective of the chapter, was to examine effects of meal size on the SDA response, at a set temperature of 17°C. Preliminary work indicated that the maximum meal size that a 48-hour fasted snapper could consume within an acceptable time frame (i.e. 5–15 minutes), was between 2.5 and 3% BM. Based on this finding the ration treatments for snapper were 0.5, 1, 2 and 3% BM. Since YEM were less cooperative in feeding trials than snapper, they only managed to be successfully fed with 0.5 and 1% BM ration. No attempt was made to feed YEM with 2% BM since introductory work suggested that a 48-hour fasted YEM would not eat more than 1.5% BM in a respirometer within the acceptable time for SDA work. The order of ration sizes at which SDA was tested was determined randomly.

4.2.2 Experimental set up and feeding protocol

During respirometry work a single fish was utilised to produce its routine metabolic rate (RMR), maximum metabolic rate (MMR) and SDA in the same block of time whenever that was feasible. This was practiced for several reasons. From an ethical point of view this would minimise manipulation and it would also increase time efficiency. After acclimation, the RMR of an individual fish was obtained. Subsequently fish were introduced into the swimming flume to undergo exhaustive exercise to produce MMR and U_{crit}. After MMR and a full recovery (i.e. within 24 hours), while still in the respirometer, the fish were fed with the pre-set meal and monitored for 48 hours.

4.2.2.1 Snapper feeding protocol

Preliminary feeding trials showed that snapper in a respirometer preferred to consume firm oily (e.g. silver trevally - *Pseudocaranx dentex*; proximate analysis) to softer fish fillets (e.g. spiny dogfish - *Squalus acanthias*). Nutritional composition of the trevally fillets according to the proximate analysis generated by Cawthron Institute (project number: T51050) was: energy 406 kJ 100 g^{-1}, crude protein 22.6 g 100 g^{-1}, total fat 0.6 g 100 g^{-1}, carbohydrate < 0.1 g 100 g^{-1}, moisture (at 105°C) 76.2 g 100 g^{-1}, and ash 1.2 g 100 g^{-1}. YEM also readily consumed trevally fillets when fed in a respirometer during the preliminary trials. Therefore, trevally fillets were chosen as the food used in the entire SDA study for both species. All fish in all SDA trials were offered a meal at 10 am. This timing was chosen for

several reasons. The sunrise period was introduced at 7 am with a gradually increasing light intensity until its peak was reached at 7:30 am. Even the slow increase of light intensity into the respirometery room would elicit increased metabolic activity in some fish. However, this metabolic activity would usually stabilise within an hour, meaning that Snapper would usually be at the RMR MO_2 level again by 9 am. Therefore the 9 am measurement was an important checking point to confirm that fish had started exhibiting RMR and could progress into the feeding trial.

As described in 3.2.1.3 the acrylic lid of the respirometer had four openings. An opening was for the oxygen electrode that was firmly sited in a rubber bung sealing the respirometer. The same opening was used for feeding snapper in all SDA trials. Just before feeding, the rubber bung with the oxygen electrode was gently removed and the meal was administrated into the respirometer. The bung was then returned into its position in the lid allowing measurements to begin. Generally, Snapper would ingest an offered meal within 1–2 minutes. In some cases, it would take 5–15 minutes for the entire meal to be consumed or fish would not consume the whole meal, as was often the case when snapper were offered the 3% BM ration. Infrequently, fish would entirely refuse to take a meal. Whenever an offered meal was not completely consumed the data were not used in the final calculation of the SDA response. The manual food administration was tested for any effects that it may pose on MO_2. A false feeding trial was conducted where all actions were carried out except no food was placed into the respirometer. The test showed that, in general, the action of removing the bung – administrating a meal – reattaching the bung did not elicit any marked increase in MO_2, if at all. However, in some cases the increase was obvious, but it would drop relatively rapidly (within $\sim$ 30 minutes) to pre-false feeding levels. To take this into account when calculating the SDA cost of Snapper, the first 30 minutes of integrated area between SDA and RMR curves were not included.

4.2.2.2 YEM feeding protocol

Mirrors were installed in the respirometer to increase YEM chances of feeding during the SDA trials. The same feeding procedure as for snapper was tested on YEM. The test revealed that manual administration of food into a respirometer did not work for YEM. The action of removing the bung – administering a meal – reattaching the bung markedly stressed YEM, the MO_2 substantially increased and consequently fish did not show any interest in food. For that reason, a 55-cm feeding tube (diameter 1 cm) with a ball valve was designed and introduced to the set-up. One end of the tube was attached to the respirometer via a new hole in the lid. The free end reached over the side of the 300 L tank where the administration of the food via the syringe could be carried out without any risk of being seen by fish. The syringe had an attachment where the finely cubed fillet meal was placed. Before the food administration and during the flushing part of the *Flush-Wait-Measure* loop the tube was rinsed and cleared of air bubbles. The syringe attachment was then connected to the ball valve

mounted at the free end of the feeding tube. Subsequently, when *Flush* turned into *Wait* the valve was opened and the food was gently pushed through the tube system into the respirometer. Water used in the syringe was taken from the 800 L resident YEM tank with the purpose of further reinforcing the deception of conspecifics in the respirometers.

4.2.3 SDA parameters description, determination and metabolic scaling

4.2.3.1 SDA parameters – description and determination

Two curves from an individual fish, one as a control baseline curve (RMR), obtained before feeding and the other the SDA MO_2 response after feeding were compared to assist in the determination of SDA variables. Twenty-four-hour RMR data were not averaged to produce a single mean value to represent a horizontal baseline against which SDA variables would be calculated. Rather, raw RMR data from each fish were preserved as a curve and matched with the corresponding hourly SDA response in a 24-hour period thus making hourly pairs. Or in other words, the pairs were constructed from MO_2 values for RMR and SDA at the same hour in a 24-hour day (i.e. RMR at 12 am vs. SDA at 12 am). Comparison started with the 10 am hourly pair when a meal was expected to be ingested. Any differences observed were assumed to be as a result of SDA. When 24-hour RMR data were not enough to measure the termination of SDA, the needed number of hours with their resting MO_2 values were added from the beginning of the RMR data to match the same hours in a 24-hour day.

The advantage of the approach of using the raw RMR data in 24-hour day as a base-curve meant any circadian effects were accounted for. Although a circadian daily pattern in resting metabolic activity was not strong and clear when RMR data from all fish at given temperature were averaged as a 24-hour resting response (Fig. 3.4, 3.6), individual fish seemed to follow a certain pattern when RMR was measured for 48 hours or more.

Eight SDA related variables were estimated in this study: SDA peak, time to peak, SDA amplitude, SDA factorial scope, SDA duration, SDA cost, SDA coefficient and limitation of AMS for activity due to SDA (%).

- SDA peak (mg O_2 kg^{-1} h^{-1}) – was the SDA MO_2 value of a RMR – SDA hourly pair with the greatest difference observed during the length of the SDA response.

- Time to peak (hours) – calculated as time elapsed until SDA peak was reached.

- SDA amplitude or absolute increase in metabolic rate (mg O_2 kg^{-1} h^{-1}) – calculated as a difference between the SDA MO_2 and RMR MO_2 value of the hourly pair at the time of SDA peak.

- SDA factorial scope – calculated as a ratio between SDA MO_2 and RMR MO_2 value of the hourly pair at the time of SDA peak.

- SDA duration (hours) – calculated as time (hours) from the beginning of SDA (meal ingested at 10 am) until when SDA MO_2 value returned within 10 % of corresponding RMR paired value for at least two consecutive hours.

- SDA cost (mg O_2 kg^{-1}; kJ) – calculated as integrated MO_2 values between RMR and SDA curves for the length of SDA duration (mg O_2 kg^{-1}). To express the cost in kJ, oxycalorific coefficient 0.01406 kJ mg O_2^{-1} (Gnaiger, 1983; Jordan and Steffensen, 2007) was utilised.

- SDA coefficient (%) – calculated as a percentage ratio between energy expended to digest a meal and energy content of the meal as described with the equation:

$$100 * \frac{SDA\ cost\ (KJ)}{Energy\ stored\ in\ a\ meal\ (KJ)}$$

  Energy content of 1g wet mass trevally fillet was 4.06 kJ.

- Limitation of aerobic metabolic scope (AS) for activity (%), as percentage of AS reduction/impairment during the SDA peak (Jordan and Steffensen, 2007), calculated with the following equation:

$$100 * \frac{SDA\ amplitude}{MMR\ at\ the\ given\ temperature - RMR\ value\ of\ the\ SDA\ amplitude}$$

4.2.3.2 Metabolic scaling to common mass

The scaling was introduced to reliably compare certain SDA parameters (i.e. SDA peak, amplitude, factorial scope, SDA cost, coefficient and limitation to AMS) between two test species. The insight about the usage of metabolic capacity, gained from estimating the impairment of AMS during the peak SDA response, pointed that up to 50% of the scope may be engaged in the response of both species.

Accordingly, the values for scaling coefficient utilised for RMR (0.74 and 0.84) and MMR (0.81 and 0.90 for snapper and YEM respectively) were averaged and scaling coefficients used specifically for the SDA data were produced (i.e. 0.78 and 0.87 for snapper and YEM respectively). For details about sources of scaling exponents refer to 3.2.3.4.

4.2.4. Statistical methods

Statistical method for intraspecific comparison of SDA variables within two treatments (i.e. temperature and ration) was the general linear model ANCOVA with fish *mass* as a covariate, unless the covariate *mass* did not significantly affect the model allowing the covariate to be dropped from the model which essentially became one-way ANOVA. ANCOVA as a statistical model was treated in the same way as ANOVA as described chapter 2. Two-way ANOVA with factors *species* and *temperature*/*ration* was employed for interspecific comparison with the primer interest in significance of the interaction term. Data for this examination was standardised by scaling to the common 100 g mass.

4.3 Results

4.3.1 Effect of temperature on SDA parameters

Estimated SDA parameter values for both species at levels of experimental temperatures and rations with statistical tests and associated p-values for comparison amongst levels of treatments within species are summarised in Tables 4.1 and 4.2 for temperature and ration treatment respectively. Snapper SDA response was tested for fish acclimated at 13, 17 and 21°C and fed with a standardised amount of feed at 0.5% body mass (BM). Set-up temperature of 17 °C was also a choice for the assessment of meal size (ration) effects on SDA response. Therefore, in addition to 0.5% at 17°C, fish were fed with rations of 1, 2 and 3% BM. This was accomplished for snapper, but not YEM, which only managed to be fed at two temperatures (i.e. 17 and 21°C) and with two rations (i.e. 0.5 and 1% BM).

4.3.1.1 Effect of temperature on snapper SDA parameters

SDA peak (SDA_{peak})

Log_{10} transformed SDA_{peak} data were different for all three experimental temperatures ($F_{2, 27}$ = 32.710, $p < 0.001$, Table 4.1). Temperature had a positive exponential effect on the peak oxygen consumption (MO_2) when fish were fed with a ration of 0.5% BM as illustrated in Fig. 4.3A.

Time to SDA peak (TTP)

In the same fashion to SDA_{peak}, TTP data had to be log_{10} transformed as violations of ANOVA assumptions were detected. Subsequently a significant effect of temperature on the time required for fish to process an ingested meal at its highest level was observed ($F_{2, 27}$ = 6.640, $p = 0.005$). The difference was identified between the SDA response at 13 and 21, and 17 and 21°C (Table 4.1). The relationship between time to SDA peak and temperature was negative and the best explanatory model was linear (Fig. 4.3B).

SDA amplitude (SDA_{amp})

SDA_{amp} had similar dynamics in relation to acclimated temperature as SDA_{peak}, where log_{10} transformed data showed exponential dependency on temperature ($F_{2, 27}$ = 15.011, $p < 0.001$, Fig. 4.3C), with the exception between the 13 and 17°C acclimated groups where no difference was found (Table 4.1).

SDA factorial scope (SDA_{fas})

SDA_{fas} changed with acclimation temperatures ($F_{2, 27}$ = 5.583, $p = 0.011$, Fig. 4.3D) but only when temperature increased from 17 to 21°C, as revealed by Holm-Sidak pairwise multiple comparison procedures (Table 4.1). Overall, there was weak or lack of relationship with temperature (Fig. 4.3D).

SDA duration (SDA_{dur})

In the same fashion to TTP, SDA_{dur} had negative linear association with temperature ($F_{2, 27}$ = 9.635, $p < 0.001$, Fig. 4.3E). However, from all three SDA_{dur} temperature combinations the only difference (due to the leverage that significant effect of covariate *mass* had on the model, $F_{1, 27}$ = 4.534, $p = 0.044$) was detected between 13 and 21°C (Table 4.1).

SDA cost (SDA_{cost})

SDA_{cost} was another SDA parameter where the general linear model (i.e. ANCOVA) was affected with differences in *mass* within the sample ($F_{1, 27}$ = 7.440, $p = 0.012$), therefore no differences between temperature acclimated groups were detected ($F_{2, 27}$ = 3.359, $p = 0.0517$, Table 4.1). Nevertheless, mean SDA_{cost} with the array of experimental temperatures, exhibited a positive exponential increase (Fig. 4.3F).

SDA coefficient (SDA_{coef})

Since SDA_{coef} is generally closely related to SDA_{cost}, the overall behaviour of the coefficient resembled the dynamics of SDA_{cost} as a function of temperatures. Therefore, the exponential model best explained the data (Fig. 4.3G). Due to lack of the significant influence on the model, covariate *mass* was dropped, and temperature was shown to have significant effect on the coefficient ($F_{2,\ 27} = 3.833$, $p = 0.035$). A difference was observed between SDA_{coef} means calculated for 13 and 21°C (Table 4.1).

Limitation of aerobic metabolic scope (AS) for activity (SDA_{limit})

Impairment of aerobic metabolic scope at the time of the highest point of MO_2 during the SDA response, due to its strong relation, clearly followed the trajectory of the SDA_{amp}/SDA_{peak} relative to temperature increase. Hence, temperature had a significant effect on the $\log_{10}$ transformed limitation data ($F_{2,\ 27} = 10.159$, $p < 0.001$) and the exponential function well explained temperature group means (Fig. 4.3H). The difference was positioned between two temperature groups, 13–21 and 17–21°C (Table 4.1).

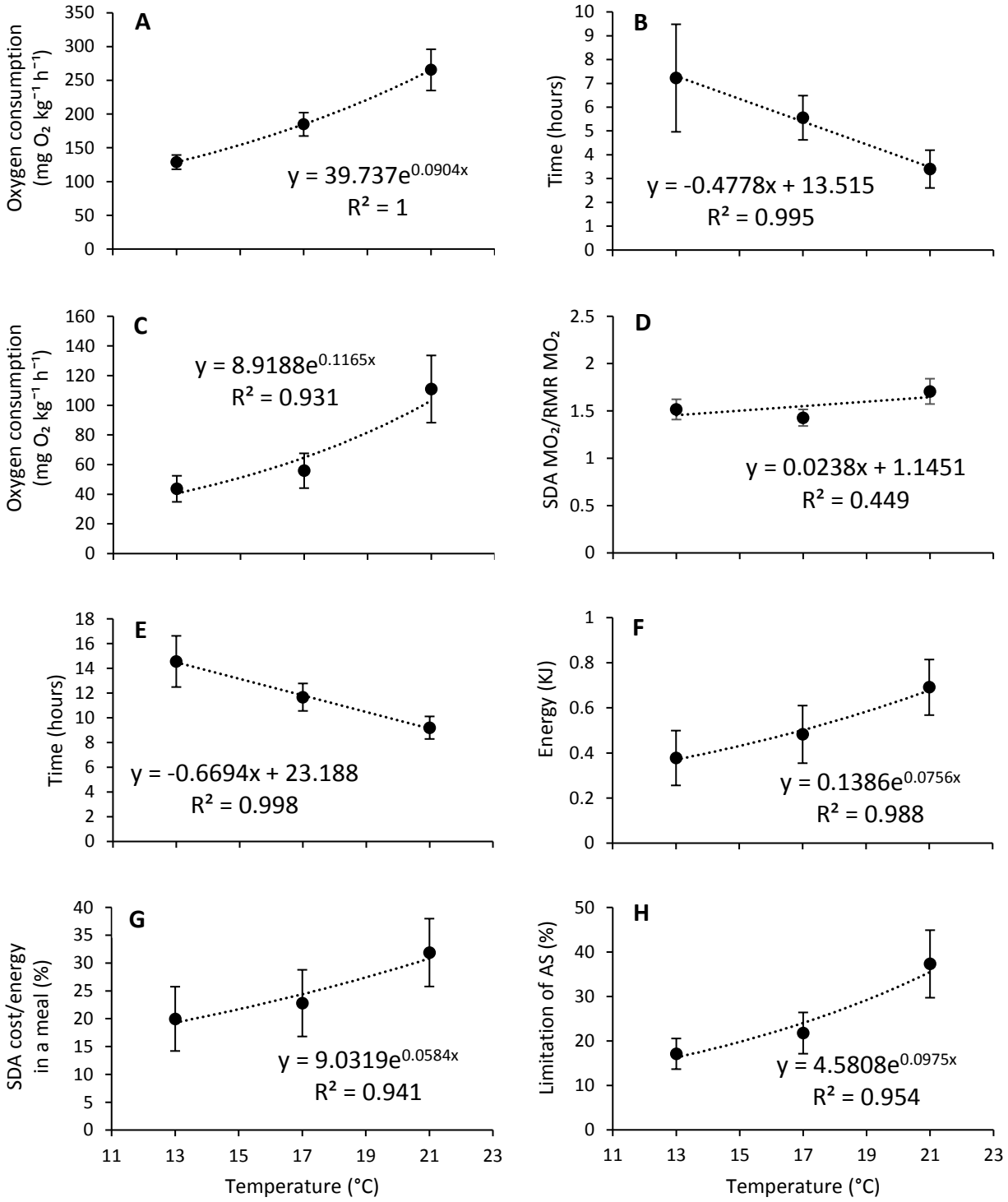

Figure 4.3. Mean SDA response of snapper fed with the set ration of 0.5% BM (body mass) as a function of experimental temperatures (i.e. 13, 17 and 21°C) for eight SDA parameters: A – peak, B – time to peak, C – amplitude, D – factorial scope, E – duration, F – SDA cost, G – SDA coefficient and H – limitation of aerobic scope for activity. Black dotted curves represent the model of the best fit described with the associated equation and R^2. Error bars are 95% confidence intervals.

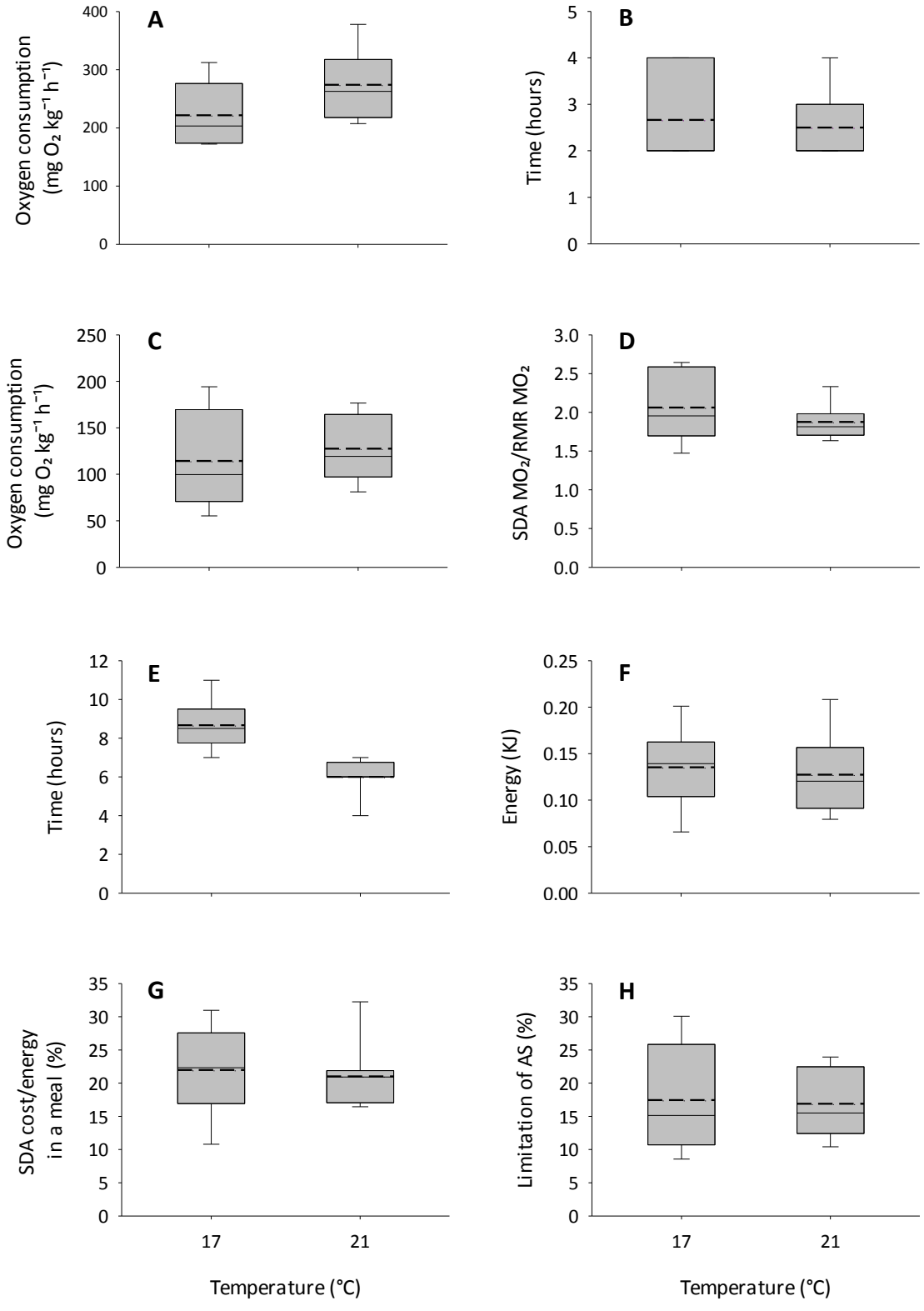

Figure 4.4. Box plot presentation of SDA response of YEM fed with the set ration of 0.5% BM (body mass) as a function of two acclimated temperatures (17 and 21°C) for eight SDA parameters: A – peak, B – time to peak, C – amplitude, D – factorial scope, E – duration, F – SDA cost, G – SDA coefficient and H – limitation of aerobic scope for activity. The ends of the boxes define the 25th and 75th percentiles, with a middle line at the median and error bars defining the 10th and 90th percentiles. Dashed lines within boxes represent mean values.

4.3.1.2 Effect of temperature on YEM SDA parameters

SDA parameters for YEM acclimated to 17 and 21°C when fed with 0.5% BM meal size were relatively stable and statistically did not change (Fig. 4.4, Table 4.1). The exception was SDA_{dur} which was significantly shorter at 21°C ($t = 4.368$, $DF = 12$, $p = 0.001$, Fig. 4.4E). This suggested that temperature did not have effects on any other aspects of energy distribution during meal digestion in YEM apart from accelerating SDA response as temperature increased from 17 to21°C.

4.3.1.3 Effect of temperature on SDA parameters – snapper versus YEM

Summary of interspecific comparison of levels of factors *temperature* and *ration* with associated p-values can be found in Table 4.3. For the comparison purpose, metabolic data of both species were scaled to a common mass of 100 g as described in 4.2.3.2.

SDA_{peak}

An output of two-way ANOVA indicated no statistical differences between test species at any two comparable experimental temperatures when fish were fed with 0.5% BM (Fig. 4.5A, Table 4.3). Furthermore, there was no interaction amongst levels of temperature treatment with factor *species*, therefore it appears that temperature had the same effects, to increase the highest level of metabolic activity due to SDA, of snapper and YEM as temperature increased from 17 to 21°C.

TTP

Unlike SDA_{peak}, time for fish to reach the maximum level of metabolic response, following a 0.5% BM meal consumption, differed for the two test species ($F_{1, 32} = 18.838$, $p < 0.001$, Fig. 4.5B, Table 4.3). Snapper required more than double the amount of time (i.e. 2.1) to reach the maximum level of oxygen consumption during digestion at 17°C, while there were no differences for the same ration at acclimated temperature of 21°C. This was aligned with the ANOVA output declaring that the interaction term between *temperature* and *species* was also statistically present ($F_{1, 32} = 5.191$, $p = 0.030$). This means that temperature, when increased from 17 to 21°C had a different impact on the time required for the two test species to reach the SDA_{peak}, in case of snapper to markedly shorten the time needed to approach to the peak, whereas for YEM the response appeared to be independent of temperature increase.

SDA_{amp}

Even though temperature influenced snapper and not on YEM SDA_{amp}, relatively small sample size accompanied with higher variability within YEM sample did not permit for the effects of main factor

species to be exposed (Table 4.3). Neither was an interaction between levels of factors detected (Fig. 4.5C).

SDA$_{fas}$

Ratio of SDA pairs (i.e. SDA MO_2 and corresponding baseline resting MO_2) during the peak postprandial response on the $\log_{10}$ transformed data indicated interspecific differences ($F_{1,\ 32}$ = 26.740, $p < 0.001$, Fig. 4.5D). The factorial scope was higher for YEM at 17°C (Table 4.3). Moreover, different effects of temperature on the scope dynamics of test species was identified as a significant interaction term between factors *species* and *temperature* ($F_{1,\ 32}$ = 6.304, p = 0.018), where the YEM's scope dropped while snapper's increased as acclimated temperature changed from 17 to 21°C.

SDA$_{dur}$

Log_{10} transformed data describing duration of SDA response of snapper and YEM at 17 and 21°C via two-way ANOVA suggested the same effects of temperature on both species (i.e. no significant interaction term observed). Nevertheless, duration of SDA response differed between test species at both experimental temperatures ($F_{1,\ 32}$ = 39.263, $p < 0.001$, Fig. 4.5E, Table 4.3), even though the general trend for the span of SDA response to shorten with increase in temperature was evident for both species.

SDA$_{cost}$

Main factor *species* was found to have a significant effect on the SDA cost ($F_{1,\ 32}$ = 8.741, p = 0.006, Fig. 4.5F). Statistical output for the comparison between the two species indicated an opposite pattern to SDA$_{fas}$ (Fig. 4.5F and 4.5D), namely there were no interspecific differences at 17°C but the difference was detected at 21 °C where the higher cost was observed for snapper (Table 4.3). However, interaction effect was not detected.

SDA$_{coef}$

Relative efficiency, expressed as SDA coefficient, of digestive capability differed between snapper and YEM when fed with 0.5 % BM ration at 17 and 21°C ($F_{1,\ 32}$ = 5.909, p = 0.021, Fig. 4.5G), with the difference observed at the higher temperature (Table 4.3). Similar to SDA$_{cost}$, an interaction effect remained undiscovered, even though group means for both parameters at two temperatures suggested that YEM responses may be independent, while snapper dependent on temperature.

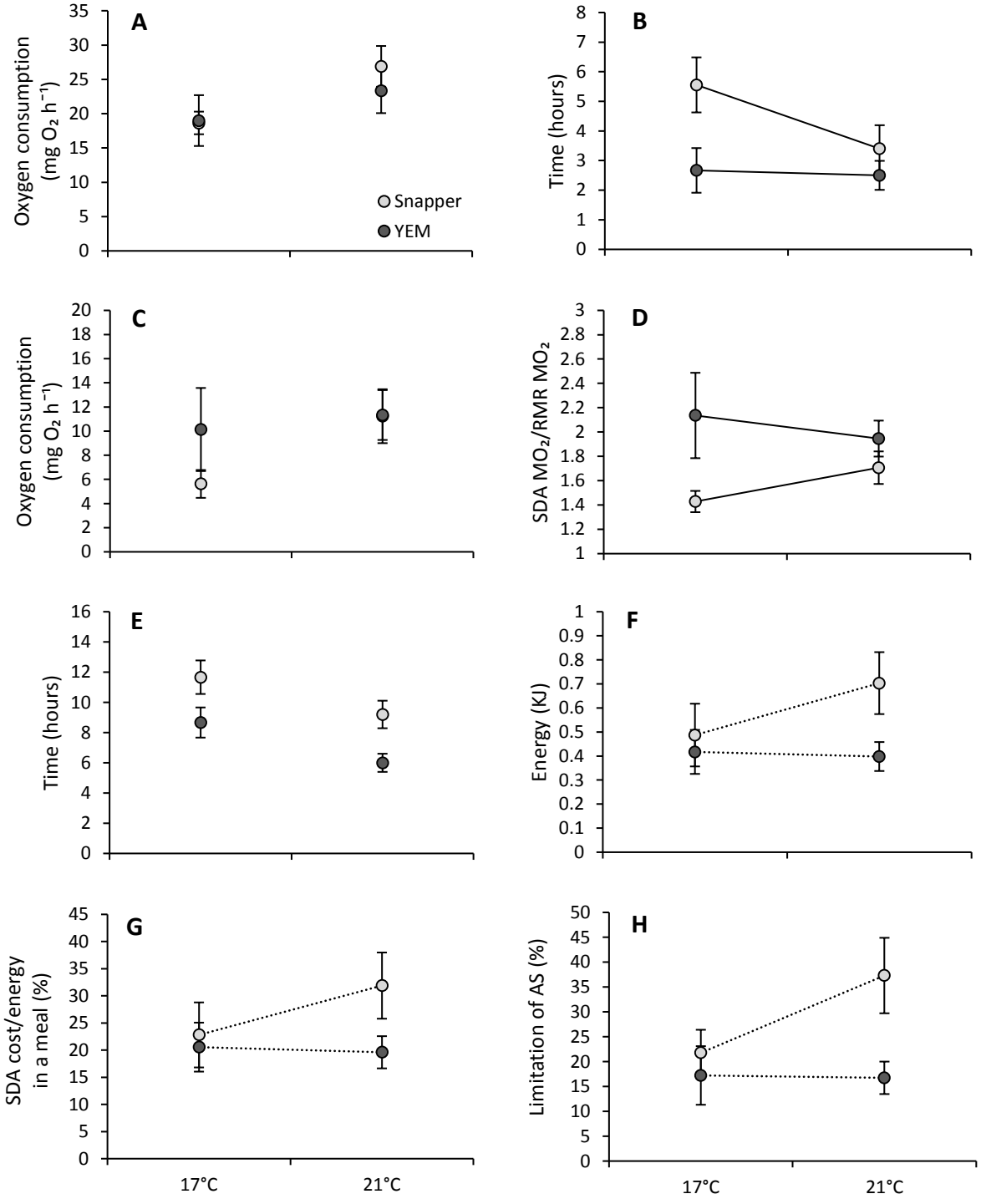

Figure 4.5. Mean SDA response of snapper (light grey symbols) and YEM (dark grey symbols) fed with the set ration of 0.5% body mass (BM) as a function of two acclimated temperatures (i.e. 17 and 21°C) for eight SDA parameters: A – peak, B – time to peak, C – amplitude, D – factorial scope, E – duration, F – SDA cost, G – SDA coefficient and H – limitation of aerobic scope for activity. Black lines connecting mean values in B and D indicate significant interactions between factors *ration* and *species* for the given SDA parameter. Black dotted lines in F, G and H indicate possible different effects of factor *temperature* on factor *species* but there were no significant levels observed (see text for details). Error bars are 95% confidence intervals.

SDAlimit

Occupancy of aerobic metabolic scope by SDA response, when compared between the two species, showed similar dynamics to the previous two parameters (SDA_{cost} and SDA_{coef}). Temperature effects were identified on $\log_{10}$ transformed data ($F_{1, 32} = 13.176$, $p = 0.001$, Fig. 4.5H), where the limitation was significantly higher for snapper at 21 °C (Table 4.3). Since YEM, contrary to snapper, did not demonstrate any changes in the parameter between the two acclimated temperatures, resembling the dynamics of SDA_{cost} and SDA_{coef}, it appears that temperature differently affected the two species. However, again, the lack of statistical power did not allow for the interaction between temperature and species to be detected ($F_{1, 32} = 3.596$, $p = 0.072$).

4.3.2 Effect of meal size on SDA parameters

4.3.2.1 Effect of meal size on snapper SDA parameters

SDApeak

The linear model that explained 63% of the data, based on mean SDA_{peak} measured at four different rations, suggested the trend of proportional increase in SDA_{peak} with the meal size (Fig. 4.6A). However, insufficient number of regression pairs (four) did not allow for the relationship to be found significant. Nevertheless, significant effects of factor *ration* via the ANCOVA model was revealed ($F_{3, 32} = 5.366$, $p = 0.006$). The differences were placed between peaks measured at 0.5 and 3, 1 and 3, and 2 and 3% BM (Table 4.2). However, since there were no differences amongst the first three rations (i.e. 0.5, 1 and 2% BM), which denoted that meal size did not have an effect in terms of maximum energy expenditure during the SDA response, the SDA_{peak} may be only affected when meals above 2% BM are ingested.

TTP

Similar to SDA_{peak}, although statistical output on $\log_{10}$ transformed data showed significant effects of meal size ($F_{3, 32} = 3.042$, $p = 0.045$), no trend was obvious in the graphical display (Fig. 4.6B). The only difference was observed between 1 and 2% BM (Table 4.2).

SDAamp

Meal size had a significant effect on SDA_{amp} ($F_{3, 32} = 6.491$, $p = 0.002$, Fig. 4.6C). Differences were detected between 0.5 and 3, and 1 and 3% BM rations (Table 4.2). The overall effect is well explained with the linear model ($R^2 = 0.92$), indicating proportional increase in SDA amplitude with increased meal size.

SDAfas

Snapper SDA_{fas} in association with change in meal size was very well explained with natural logarithmic model (R^2 = 99, Fig. 4.6D). In addition, the change in ration size significantly affected the scope ($F_{3, 32}$ = 6.755, p = 0.0012), with differences detected between 0.5 % with both higher (i.e. 2 and 3 % BM) rations (Table 4.2).

SDA_{dur}

In comparable fashion to SDA_{amp}, the relationship of SDA_{dur} with the set of experimental rations was positive and well explained with the linear model (R^2 = 0.93, Fig. 4.6E). Besides, meal size had a significant effect on duration of the SDA response (log_{10} transformed data, $F_{3, 32}$ = 33.187, p < 0.001). The differences were between the lower ration groups (i.e. 0.5 and 1% BM) and with each of the two higher ration groups (i.e. 2 and 3% BM) (Table 4.2). Despite good explanation of data with linear model, since there were no differences between means of two lower and two higher rations groups and variability within groups was found to be relatively stable, the effect of meal size appeared to be stepwise rather than linear (black lines in Fig. 4.6E)

SDA_{cost}

In terms of statistical output and the model that best fitted the data, SDA_{cost} was similar to the SDA_{dur} (log_{10} transformed data, $F_{3, 32}$ = 15.643, p < 0.001, R^2 = 0.98, Fig. 4.6F, Table 4.2). This was not surprising since the two parameters are closely related.

SDA_{coef}

Efficiency of snapper to digest and assimilate ingested food did not change according to meal size, as demonstrated with One-way ANOVA on ranks ($F_{3, 32}$ = 0.804, p = 0.509, Fig. 4.6G, Table 4.2). Nevertheless, natural logarithmic function explained 53% of data and it appeared that there was a general tendency to decrease with meal size (Fig. 4.6G), indicating that ration may have an effect of the coefficient; however, it may not be a simple function of meal size.

SDA_{limit}

Change in ration size influenced how much aerobic metabolic scope was taken at the point of SDA_{peak} ($F_{3, 32}$ = 7.556, p < 0.001, Fig. 4.6H). Since SDA_{peak} and SDA_{limit} are not independent of each other, their dynamics in relation to meal size were similar. Therefore only 3% ration differed to all lower rations (Table 4.2). The difference between the two parameters was that the SDA_{limit} was better explained with the exponential model (Fig. 4.6H).

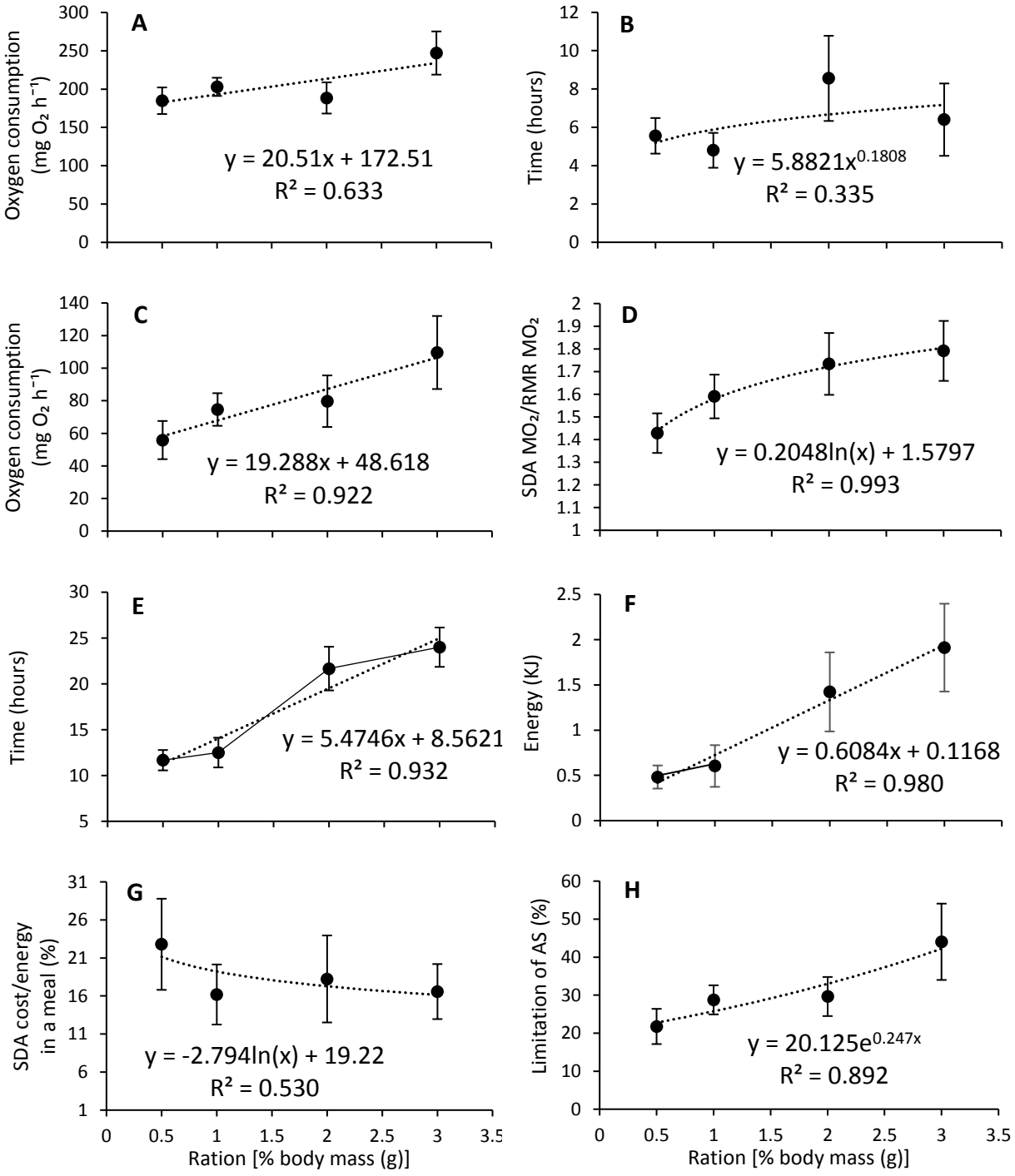

Figure 4.6. Mean SDA response of snapper acclimated to set temperature of 17°C and fed with four rations (i.e. 0.5, 1, 2 and 3% body mass) for eight SDA parameters: A – peak, B – time to peak, C – amplitude, D – factorial scope, E – duration, F – SDA cost, G – SDA coefficient and H – limitation of aerobic scope for activity. To illustrate the lack of effects of meal size on SDA duration at lower (0.5, 1% BM; E, F) and higher rations (1, 2% BM; E), but clear differences between lower and higher ration groups in the step-wise fashion (E), black lines connecting rations were introduced (for explanation see text). The rest of the figure caption is the same as for Fig. 4.3.

4.3.2.2 Effect of meal size on YEM SDA parameters

Similar to temperature effects, effects of meal size were negligible for many SDA parameters including SDA_{peak}, SDA_{amp}, SDA_{fas}, SDA_{coef} and SDA_{limit} (Fig. 4.7, Table 4.2). Even though statistical difference was also not evident for the rest of the parameters (i.e. TTP, SDA_{dur} and SDA_{cost}) the nature of the data required the results to be interpreted with caution. The general appearance that meal size had overall smaller effect on YEM than snapper SDA response, may be just an artefact of restriction to test YEM at 0.5 and 1% BM rations only. With closer examination the opposite became evident, since there were no differences between 0.5 and 1% BM ration for any snapper SDA parameters.

TTP

Mean values for time needed to reach the maximum postprandial metabolic performance for YEM fed with 0.5 and 1% BM ration were found to be nearly significantly different ($F_{1, 11} = 4.673$, $p = 0.056$). The statistical output specified that the power of the performed test (i.e. 0.40) was below the statistically desirable level of 0.80. This was due to a relatively low sample size ($n = 6$) and its associated variability. If only means are concerned (i.e. 2.67 ± SD 1.03 and 6.00 ± SD 3.63 hours for 0.5 and 1% BM ration respectively) it is apparent that an increased ration was followed on average with longer time to reach the SDA_{peak}. Therefore, the notion that ration may have had an effect on the SDA_{peak} of YEM cannot be entirely ruled out.

SDA_{dur} and SDA_{cost}

Both SDA parameters when presented graphically (Fig. 4.7E and 4.7F) or just by observing their descriptive statistics (i.e. SDA_{dur} = 8.67 ± SD 1.25 and 13.83 ± SD 3.02 hours for 0.5 and 1% BM; and SDA_{cost} = 0.14 ± SD 0.04 and 0.36 ± SD 0.13 KJ for 0.5 and 1% BM respectively) appear to be different within the ration treatment. That would be true if it was not for the effect of covariate *mass* on the model performance (i.e. $F_{1, 11} = 18.555$, $p = 0.002$ and $F_{1, 11} = 17.676$, $p = 0.002$ for SDA_{dur} and SDA_{cost} respectively). Only one additional data point may overbalance or strengthen the *mass* effect, therefore as for the time to SDA_{peak}, SDA_{dur} and SDA_{cost} should be interpreted cautiously.

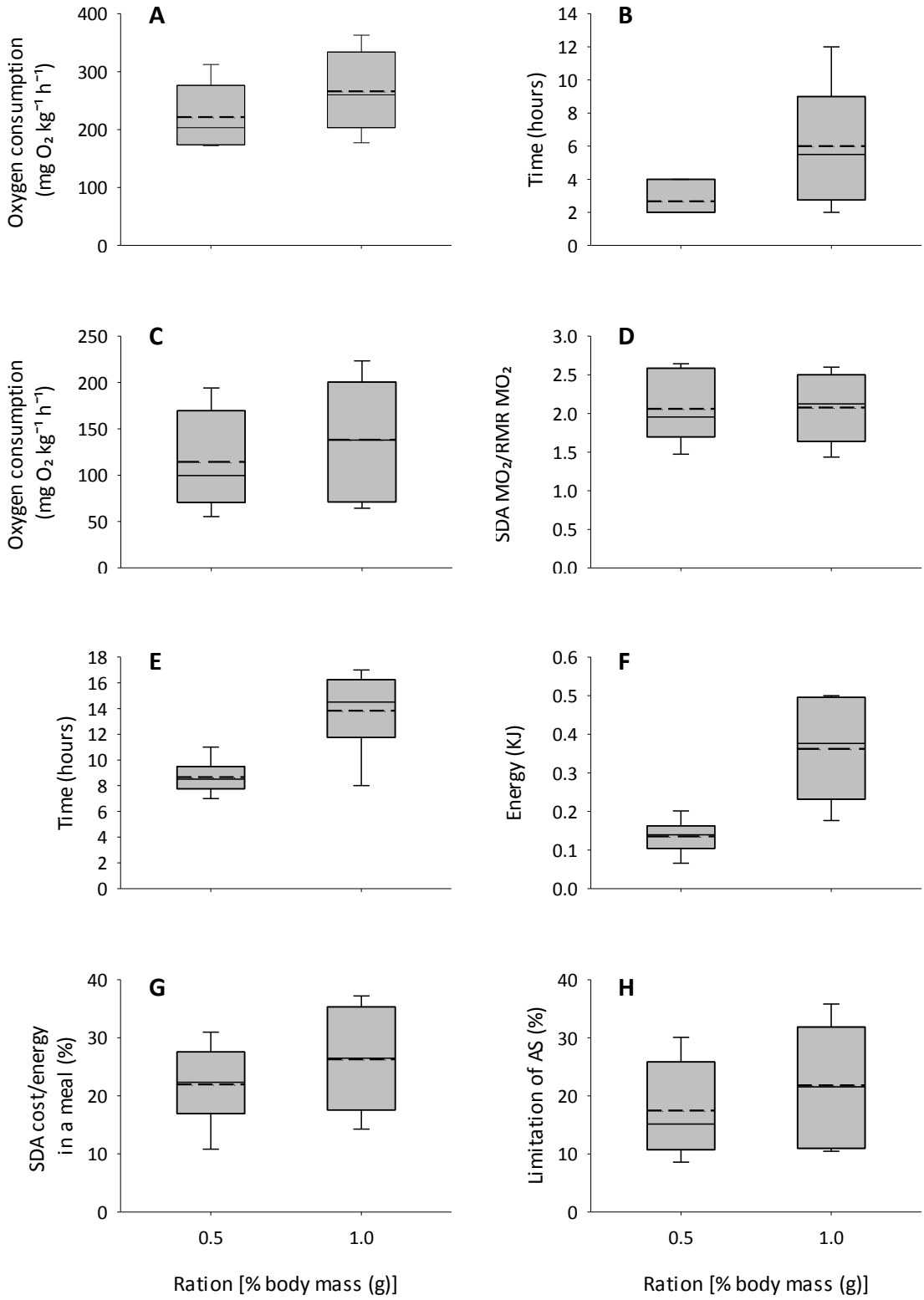

Figure 4.7. Box plot presentation of SDA response of YEM fed with the set ration of 0.5% BM (body mass) as a function of two acclimated temperatures (17 and 21°C) for eight SDA parameters: A – peak, B – time to peak, C – amplitude, D – factorial scope, E – duration, F – SDA cost, G – SDA coefficient and H – limitation of aerobic scope for activity. The rest of the figure caption is the same as for Fig. 4.4.

4.3.2.3 Effect of meal size on SDA parameters – snapper versus YEM

SDA_{peak}

Two-way ANOVA output of SDA_{peak} data obtained for fish fed at 0.5 and 1% BM scaled to the common mass of 100 g and transformed by the inverse approach (1 $data^{-1}$) did not expose any difference between the two species ($F_{1, 30}$ = 0.403, p = 0.5310, Fig. 4.8A, Table 4.3). Neither the interaction between factors *species* and *ration* was detected, therefore there was also no indication that meal size had an effect on SDA_{peak} which was depended on a species tested.

TTP

The statistical output described that there were no differences between species for the two ration levels ($F_{1, 30}$ = 1.273, p = 0.269). However, due to pronounced interaction effects ($F_{1, 30}$ = 7.459, p = 0.011), which signified that the manner how meal size affected the SDA parameter in question, depended on species (i.e. snapper response dropped while YEM response increased with change of the ration from 0.5 to 1% BM), the difference between snapper and YEM at 0.5% BM ration remained obscured (Fig. 4.8B). The difference, at 0.5% ration, became evident when post-hoc pairwise comparison test was performed (Table 4.3).

SDA_{amp}

Log_{10} transformed SDA_{amp} data were significantly different between the two test species ($F_{1, 30}$ = 17.365, p < 0.001, Fig. 4.8C). The difference was observed when fish were fed with both 0.5 and 1% BM ration (Table 4.3). The absence of a significant interaction term states that the effect of different levels of ration did not depend on whether snapper or YEM were offered the meal.

SDA_{fas}

To investigate if there were any differences between species, the raw data had to be inversely transformed before a statistical test was performed. A two-way ANOVA demonstrated overall differences between the two species ($F_{1, 30}$ = 32.953, p < 0.001, Fig. 4.8D). After the post-hoc test the difference was found at 0.5% BM ration (Table 4.3). There were no interaction effects, indicating that the change in ration had generally the same effect on both test species.

SDA_{dur}

In the same fashion to TTP, statistical output of SDA_{dur} detected the interaction effect ($F_{1, 30}$ = 5.869, p = 0.022, Fig. 4.8E). Therefore, levels of meal size had different effects on test species – snapper response did not change, while YEM exhibited increase in mean SDA_{dur} from 8.67 to 13.83 hours. Identical to TTP, initial two-way ANOVA output did not indicate any differences between species ($F_{1, 30}$ = 0.868, p = 0.360). Nevertheless, after Holm-Sidak all pairwise multiple comparison procedures, the difference at the ration level of 0.5% BM was evident (Table 4.3, Fig. 4.8D).

SDA$_{cost}$

Comparable to the previous two SDA parameters (namely TTP and SDA$_{dur}$), after processing $\log_{10}$ transformed data, two-way ANOVA table did not reveal differences between two species in energy expenditure as a response to ingested meal either at 0.5 or 1% BM ration ($F_{1, 30}$ = 1.929, p = 0.176, Fig. 4.8F). The same argument as for TTP and SDA$_{dur}$ could be applied, since a significant difference was observed after post-hoc test at ration level of 1% BM (Table 4.3). However, more importantly there was significant interaction effect ($F_{1, 30}$ = 4.229, p = 0.050). Therefore, it was demonstrated that an overall effect of change in ration size on the extent of the SDA$_{cost}$ depended on species, because there were no effects on snapper (i.e. no difference in SDA$_{cost}$ between two rations) but YEM was affected (i.e. SDA$_{cost}$ increased when meal size changed from 0.5 to 1% BM).

SDA$_{coef}$

SDA coefficient, on the other hand, was statistically unchanged for both, interaction effect ($F_{1, 30}$ = 3.110, p = 0.089) and differences between the two species ($F_{1, 30}$ = 1.010, p = 0.324, Fig. 4.8G, Table 4.3).

SDA$_{limit}$

Similar to SDA$_{coef}$, two-way ANOVA indicated that there was no interaction between factors *ration* and *species* and no difference between species for any level of ration (Fig. 6 H; $F_{1, 30}$ = 2.869, p = 0.102; $F_{1, 30}$ = 0.003, p = 0.957 for interaction term and species differences respectively).

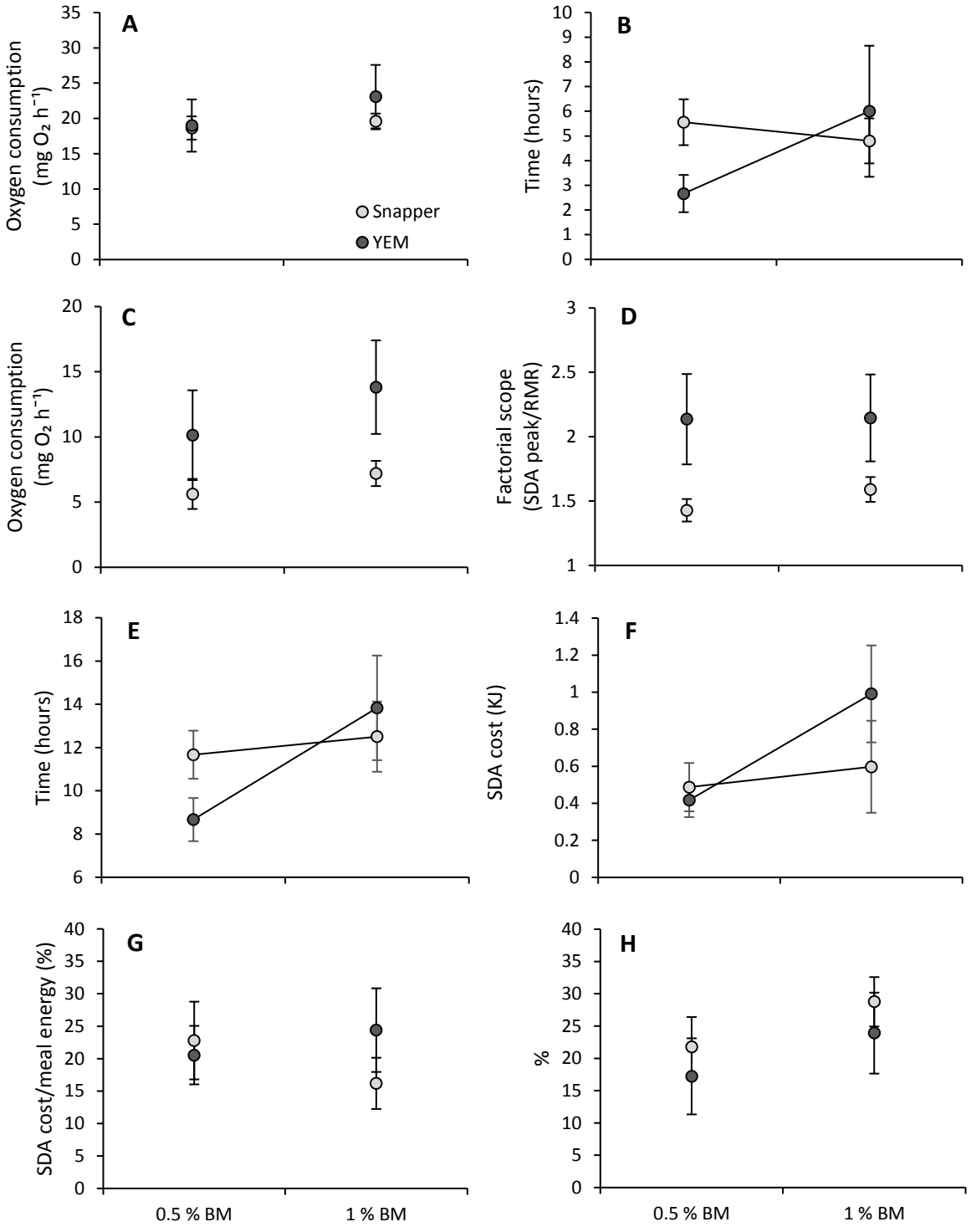

Figure 4.8. Mean SDA response of snapper (light grey symbols) and YEM (dark grey symbols) acclimated to set temperature of 17°C and fed with two rations (i.e. 0.5 and 1% body mass) for eight SDA parameters: A – peak, B – time to peak, C – amplitude, D – factorial scope, E – duration, F – SDA cost, G – SDA coefficient and H – limitation of aerobic scope for activity. Black lines connecting mean values in B, E and F indicate significant interactions between factors *ration* and *species* for the given SDA parameter. Error bars are 95% confidence intervals.

Table 4.1. Specific dynamic action (SDA) parameters estimated for snapper and YEM with sample size and descriptive statistics of mass of fish fed with set ration of 0.5% body mass (BM) at three (13, 17 and 21°C) and two (17 and 21°C) acclimated temperatures for snapper and YEM respectively – left boxes; statistical tests (for snapper data: ANCOVA (analysis of covariance), proceeded when covariate *mass* had an effect, One-Way ANOVA (analysis of variance), proceeded when covariate *mass* dropped out of the ANCOVA model due to the lack of the effect; for YEM data: ANCOVA and Student t–test, employed in the same manner as for snapper) with associated p-values generated from testing the mean parameter values within the temperature treatment – right boxes. P-values in bold and italic represent significant (i.e. ≤ 0.05) and non-significant differences (i.e. > 0.05) respectively.

	Snapper			YEM					
TEMPERATURE	13°C	17°C	21°C	17°C	21°C		ANCOVA/		ANCOVA/
Set Ration (0.5 % BM)							One-Way ANOVA		Students t
N	9	9	10	6	8		Snapper		YEM
Mass (g)	91.6 ± 7.1	105.3 ± 10.1	107.5 ± 15.6	30.3 ± 1.3	29.4 ± 3.9	13-17°C	13-21°C	17-21°C	17-21°C
SDA parameters							Between temperature p-values		
SDA peak (mg O_2 kg^{-1} h^{-1})	128.8 ± 16.2	184.8 ± 26.6	266.5 ± 49.3	221.6 ± 53.0	274.2 ± 55.0	**0.022**	**< 0.001**	**0.001**	*0.122*
Time to SDA peak (h)	7.2 ± 3.5	5.6 ± 1.4	3.4 ± 1.3	2.7 ± 0.9	2.5 ± 0.7	*0.472*	**0.006**	**0.025**	*0.732*
Amplitude (mg O_2 kg^{-1} h^{-1})	43.7 ± 13.5	55.9 ± 17.9	110.9 ± 36.6	114.4 ± 50.0	127.6 ± 33.8	*0.226*	**< 0.001**	**0.001**	*0.595*
Factorial scope	1.5 ± 0.2	1.4 ± 0.1	1.7 ± 0.2	2.1 ± 0.4	1.9 ± 0.2	*0.329*	*0.069*	**0.01**	*0.339*
Duration (h)	14.6 ± 3.2	11.7 ± 1.7	9.2 ± 1.5	8.7 ± 1.2	6.0 ± 0.9	*0.227*	**0.002**	*0.11*	**< 0.001**
SDA cost (KJ)	0.38 ± 0.19	0.48 ± 0.20	0.69 ± 0.20	0.14 ± 0.04	0.13 ± 0.04	*0.999*	*0.114*	*0.114*	*0.744*
SDA coefficient (%)	20.0 ± 9.0	22.8 ± 9.2	31.8 ± 9.8	22.0 ± 6.3	21.0 ± 4.7	*0.549*	**0.043**	*0.108*	*0.773*
SDA AS limitation (%)	17.1 ± 5.3	21.8 ± 7.1	37.3 ± 12.2	17.5 ± 7.7	16.9 ± 4.9	*0.258*	**< 0.001**	**0.008**	*0.998*

Table 4.2. Specific dynamic action (SDA) parameters estimated for snapper and YEM with sample size and descriptive statistics of mass of fish acclimated to set temperature of 17°C fed with four (0.5, 1, 2 and 3% BM) and two (0.5 and 1% BM) rations for snapper and YEM respectively – left boxes; statistical tests (for snapper data: ANCOVA, proceeded when covariate *mass* had an effect, One-Way ANOVA, proceeded when covariate *mass* dropped out of the ANCOVA model due to the lack of the effect for snapper; for YEM data: ANCOVA and Student t–test, employed in the same manner as for snapper) with associated p-values generated from testing the mean parameter values within the ration treatment – right boxes. p-values in bold and italic represent significant (i.e. ≤ 0.05) and non-significant differences (i.e. > 0.05) respectively.

	Snapper				YEM		ANCOVA/ One-Way ANOVA						ANCOVA/ Students t
RATION	0.5 % BM	1 % BM	2 % BM	3 % BM	0.5 % BM	1 % BM							
Set Temperature (17°C)							Snapper						YEM
N	9	10	9	5	6	6	0.5 - 1% BM	0.5 - 2% BM	0.5 - 3% BM	1 - 2% BM	1 - 3% BM	2 - 3% BM	0.5 - 1% BM
Mass (g)	105.3 ± 10.1	87.1 ± 19.7	98.7 ± 14.0	93.4 ± 6.5	30.3 ± 1.3	33.6 ± 2.4							
SDA parameters							Between ration p-values						
SDA peak (mg O_2 kg^{-1} h^{-1})	184.8 ± 26.6	203.0 ± 19.2	188.5 ± 31.2	247.0 ± 32.2	221.6 ± 53.0	266.1 ± 64.8	*0.449*	*0.789*	**0.009**	*0.484*	**0.019**	**0.008**	*0.262*
Time to SDA peak (h)	5.6 ± 1.4	4.8 ± 1.5	8.6 ± 3.4	6.4 ± 2.2	2.7 ± 0.9	6.0 ± 3.3	*0.58*	*0.26*	*0.65*	**0.035**	*0.625*	*0.557*	*0.056*
Amplitude (mg O_2 kg^{-1} h^{-1})	55.9 ± 17.9	74.6 ± 16.1	79.7 ± 24.2	109.6 ± 25.6	114.4 ± 50.0	138.3 ± 58.8	*0.142*	*0.084*	**< 0.001**	*0.619*	**0.035**	*0.083*	*0.503*
Factorial scope	1.4 ± 0.1	1.6 ± 0.2	1.7 ± 0.2	1.8 ± 0.2	2.1 ± 0.4	2.1 ± 0.4	*0.147*	**0.004**	**0.004**	*0.188*	*0.143*	*0.552*	*0.954*
Duration (h)	11.7 ± 1.7	12.5 ± 2.6	21.7 ± 3.7	24.0 ± 2.5	8.7 ± 1.2	13.8 ± 3.0	*0.475*	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	*0.466*	*0.121*
SDA cost (KJ)	0.48 ± 0.20	0.60 ± 0.37	1.42 ± 0.67	1.91 ± 0.55	0.14 ± 0.04	0.36 ± 0.13	*0.46*	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	*0.396*	*0.091*
SDA coefficient (%)	22.8 ± 9.2	16.2 ± 6.4	18.2 ± 8.7	16.6 ± 4.1	22.0 ± 6.3	26.3 ± 9.2	*> 0.05*	*> 0.05*	*> 0.05*	*> 0.05*	*> 0.05*	*> 0.05*	*0.396*
SDA AS limitation (%)	21.8 ± 7.1	28.8 ± 6.2	29.6 ± 7.9	44.0 ± 11.5	17.5 ± 7.7	21.8 ± 9.5	*0.154*	*0.16*	**< 0.001**	*0.822*	**0.012**	**0.018**	*0.93*

Table 4.3. Two-Way ANOVA p-values for comparison of SDA parameters between snapper and YEM scaled to the common mass of 100 g within temperature (17 and 21°C) and ration (0.5 and 1% BM) treatments as depicted in Fig. 4.5 and 4.8. p-values in bold and italic represent significant (i.e. ≤ 0.05) and non-significant differences (i.e. > 0.05) respectively.

	TEMPERATURE		RATION	
	Snapper vs. YEM		Snapper vs. YEM	
	Two - Way ANOVA			
	17°C	21°C	0.5 % BM	1 % BM
SDA parameters	**p-values**		**p-values**	
SDA peak (mg O_2 kg^{-1} h^{-1})	*0.886*	*0.113*	*> 0.05*	*> 0.05*
Time to SDA peak (h)	**< 0.001**	*0.134*	**0.012**	*0.262*
Amplitude (mg O_2 kg^{-1} h^{-1})	**0.018**	*0.953*	**0.009**	**0.004**
Factorial scope	**< 0.001**	*0.056*	**< 0.001**	**0.003**
Duration (h)	**0.002**	**< 0.001**	***0.026***	*0.296*
SDA cost (KJ)	*0.464*	**0.001**	*0.671*	**0.019**
SDA coefficient (%)	*0.617*	**0.005**	*> 0.05*	*> 0.05*
SDA AS limitation (%)	*0.246*	**< 0.001**	*0.261*	*0.222*

4.4 Discussion

4.4.1 SDA observation

In all instances of investigating SDA response after ingesting a meal (temperature or ration treatments), both test species demonstrated a typical response curve with a rapid initial increase in metabolic rate. The SDA peak (SDA_{peak}) in oxygen consumption (MO_2) was reached between 2 and 9 hours, followed by a gradual decrease until metabolic rates returned to their pre-feeding levels between 6 and 24 hours postprandial (depending on treatment). Those values were within ranges commonly reported for other fish species and ectotherms as reviewed in McCue (2006) and Secor (2009). Overall, snapper appeared to be more responsive to treatments than YEM; however, this may just reflect the lack of success in obtaining YEM data for all levels of treatments due to difficulties associated with YEM feeding in the respirometer. Nevertheless, temperature was a strong modulator of snapper SDA profile where all parameters measured responded with a clear pattern, except for SDA factorial scope (SDA_{fas}), whereas YEM had only SDA duration (SDA_{dur}) significantly shortened when temperature increased from 17 to 21°C. Similarly, in terms of meal size, all aspects of snapper SDA were affected. But, differences in SDA parameters between 0.5 and 1% BM (body mass) rations (i.e. only the rations that YEM were successfully fed) were not detected for both snapper and YEM. However, YEM ration data should be interpreted with care since low statistical power and/or fish mass effects on data did not permit statistical detection of differences in the time to SDA peak (TTP), SDA_{dur} and SDA cost (SDA_{cost}) as suggested by mean values alone (Table 4.2, Fig. 4.7). Nevertheless, interspecific comparison revealed that differences due to temperature were observed for all SDA parameters, apart from SDA peak, while dissimilarities due to meal size were detected for fewer number of tested parameters between the two model species.

In this study, size (length) of utilised fish were matched with the respirometer in such a way that free movement (comfortable 360°C turning) was allowed. Additional activity of the subjects was particularly common during the act of food handling and ingestion, especially in the case of YEM. Since it is difficult to discern between energy used for the feeding act (i.e. mechanical SDA energy costs) from the energy utilised for the actual food processing and assimilation (i.e. biochemical SDA costs) as a major SDA energy carrier (Whiteley et al., 2001; McCue et al., 2005), many authors facing the same circumstance used a term *apparent* SDA (e.g. Jobling, 1981b; Guinea and Fernandez, 1997). Therefore, *apparent* SDA may better depict the condition of the metabolic response due to feeding in the current work; nevertheless, the abbreviated version (SDA) has been employ throughout the thesis.

4.4.1.1 Effects of temperature on SDA response

When a species in a study is of a potential commercial value an investigator may first want to find out at what temperature a rate of meal turnover (i.e. SDA_{dur}) is optimised (i.e. shortest) and how this is associated with TTP and SDA_{peak}. These SDA parameters are often found to correlate, SDA_{dur} and TTP negatively (i.e. enabling more frequent feeding) and the SDA_{peak} positively with the rate of protein anabolism and ultimately growth (Jobling, 1981b; Luo and Xie, 2008). Equally important is the calculation of energetic costs of SDA (SDA_{cost}) and the percentage of energy in a meal allocated to its processing and assimilation (i.e. the SDA coefficient, SDA_{coef}). This will assist in evaluating the amount of energy available for other activities and when linked with the temperature at which digestion of a meal is most efficient, as represented with the lowest SDA_{coef}, optimal thermal zone for SDA can be determined (Tirsgaard et al., 2015).

TTP

TTP represents time elapsed before the highest level of aerobic cellular activity required to support postprandial biochemical reactions is reached. In this study YEM reached SDA_{peak} within 2 hours and this time period appeared to be independent of temperature, while snapper had a typical inverse response in TTP to increased temperatures, as commonly reported (Pirozzi and Booth, 2009; Pang et al., 2010; Vanella et al., 2010). The TTP/temperature relationship observed for snapper in the present study was contrary to findings of Guinea and Fernandez (1997) where a kin species *Sparus aurata* had a variable response without a defined pattern. The lack of clarity in Guinea and Fernandez (1997) could possibly be due to a relatively low statistical power (n = 3) and variable fish size used (40–100g), since size has also been found to affect SDA response (Peck et al., 2005; Pirozzi and Booth 2009). As mentioned, YEM in this study did not show temperature effects, but TTP of comparable sized leaping mullet (*Liza saliens*) decreased with increased temperature (i.e. 15–20 - 25°C) (Guinea and Fernandez, 1991). However, caution should be employed when interspecific comparisons are carried out even within related species of similar size particularly when feed type/composition and ration size are not matching (Secor, 2009). In Guinea and Fernandez (1991) 2.6% BM formulated pellets were used in contrast to the current study where feed was 0.5% BM wet trevally (*Pseudocaranx dentex*) muscle. However, the relatively low ration used in the current study and the fact that SDA was obtained only for two temperatures may confound detection of temperature effects on YEM TTP.

SDA_{peak}, SDA_{dur}

It is considered that the higher the SDA_{peak} the higher the rate of protein accretion, and the shorter the SDA duration the sooner is feeding able to be reinstated (Jobling, 1981b, Luo and Xie, 2008). Temperature range extended over the thermal comfort zone affects SDA_{peak} and SDA_{dur} in a way that

the two parameters are often negatively correlated (McCue, 2006). This implies that the rise in temperature may affect both parameters in favour of enhanced growth, and also that the resulting net energy expenditure could stay more or less unchanged between temperatures (Wang et al., 2002; Secor et al., 2007; Luo and Xie, 2008; Frisk et al., 2013). Thus, it is often reported that temperature has little effect on SDA_{cost} and related SDA_{coef} (McCue, 2006, Secor, 2009). This inverse SDA_{peak}/SDA_{dur} relationship as a function of temperature was observed for both test species in the present study. However, since snapper SDA_{peak} increased exponentially and SDA_{dur} decreased linearly net SDAcost also increased with temperature. Similar to snapper, *S. aurata* demonstrated negative SDA_{peak}/SDA_{dur} association with positively affected SDA_{cost} as temperature increased (Guinea and Fernandez, 1997). The SDA_{cost} increased with the rise in temperature also in rockcod (*Paranotothenia magellanica*), southern cod (*Patagonotothen sima*), magellan plunderfish (*Harpagifer bispinis*), eelpout (*Austrolycus depressiceps*) (Vanella et al., 2010), southern catfish (*Silurus meridionalis*) (Luo and Xie, 2008), and hapuku (*Polyprion oxygeneios*) (Khan et al., 2015). In contrast to snapper, in YEM and some other mullets (i.e. thicklip mullet *Chelon labrosus*, Flowerdew and Grove, 1980; and *L. saliens*, Guinea and Fernandez, 1991) temperature had no effect on change in SDA_{cost} as was observed in many other species such as in haddock (*Melannogramus aeglefinus*), Atlantic cod (*Gadus morhua*) (Perez-Casanova et al., 2010), mulloway (*Argyrosomus japonicas*) (Pirozzi and Booth, 2009), and pikeperch (*Sander lucioperca*) (Frisk et al., 2013).

At 13°C snapper data illustrated two distinct peaks in the postprandial MO_2, occurring ~1 and ~4 hours after feeding. Similarly, Luo and Xie (2008) working on southern catfish (*S. meridionalis*) observed a second SDA peak in fish exposed to a low threshold temperature for growth (i.e. 17.5°C). Luo and Xie (2008) proposed that the reason for this occurrence may be a result of partial separation of mechanical, which commonly begins immediately upon food ingestion, from biochemical SDA processes, which at lower temperatures may start with an observable lag time. In addition, several investigations conducted on Antarctic fish taxa also reported a secondary peak during the SDA response which was likewise associated with the lower thermal boundary (Austin, 2015) and/or small ration (i.e. 2.5% BM in Boyce and Clarke, 1997, where the second peak was not present in fish fed to satiation, which was equal to 14% BM, coinciding with the present study where 0.5% BM ration was used). However, the processes depicted in the second peak of temperate species (Luo and Xie, 2008 and the present study) and Antarctic species may not be the same since it is unlikely that the lag time for biochemical SDA response of approximately 100 hours postprandial observed in Antarctic species was plausible but rather it can reflect aerobic activity of certain components of biochemical SDA response such as urea production and excretion, as suggested by Clarke and Prothero-Thomas (1997), and Boyce and Clarke (1997).

SDAcoef, SDAcost

SDAcoef (%) is considered one of the most informative SDA variables since it corrects for variation in energy intake (Tirsgaard et al., 2015) and provides insights into energy efficiency during digestion under different experimental conditions. However, since there is nonlinearity of SDA responses that stem from variable meal sizes, different masses of tested organisms (Beaupre, 2005), or because of factors known to directly affect SDAcoef such as composition of meal, apparent digestibility coefficient (ADC) and differences in experimental design (Jordan and Steffensen 2007; Behrens et al. 2012) any direct comparisons of data between studies are not recommended. However, trends and a general broad comparison of results among studies are still possible (McCue, 2006; Tirsgaard et al., 2015). SDAcoef due to differences amongst species and experimental conditions showed a wide range of values in the literature spanning from 1.6% as reported for rainbow trout (*Oncorhynchus mykiss*) (Smith et al., 1978) to 59% as calculated for stone moroko (*Pseudorasbora parva*) (Cui and Liu, 1990). The higher SDAcoef found in the present work (i.e. 16 – 30%) compared with the average across fish studies (~15%) was possibly due to the use of natural food (trevally fillets), which is usually associated with a higher SDAcoef (i.e.> 25 %) than a formulated pellet diet with low SDAcoef (< 5 %) (Secor, 2009). Snapper SDAcoef appeared to be positively related to temperature (with higher SDAcoef representing lower energy efficiency), but YEM SDAcoef was unaffected as was also reported for thicklip mullet (Flowerdew and Grove, 1980). Guinea and Fernandez (1997) working with similar sized *S. aurata* to the present study snapper, also demonstrated that temperature had a negative impact on digestive efficiency of a meal. Despite utilising feed with higher energetic content (pellets) compared with trevally fillet in the current study (i.e. on average commercial pellets contain around five times more energy than natural food such as fish fillets), for similar ration size, SDAcoef values were highly comparable between the two studies. This may suggest that the change in feed type does not affect the proportion of energy devoted to meal processing relative to energy content of the meal at any given temperature for sparids (at least for 16/17 and 21°C as shown for *C. auratus* and *S. aurata* in the two studies).

Although temperature is generally expected to have a small impact on SDAcost and SDAcoef (McCue, 2006; Secor, 2009), as described earlier, SDAcoef has been found in many instances to increase with temperature, but this does not necessarily mean that the highest efficiency as depicted with the lowest SDAcoef often found at the lowest temperature tested is factual (Tirsgaard et al., 2015). Tirsgaard and colleagues (2015) suggested that the lower SDAdur, SDAcost and SDAcoef observed in *Gadus morhua* at the lowest experimental temperature (2°C) compared with 4°C was actually the consequence of a low apparent digestibility coefficient (ADC). Decline in ADC at low temperatures is believed to mirror impaired activities of gut proteases (Kofuji et al., 2005) followed by partial digestion of a meal as it passes through the body (Jobling, 1994). This disturbed digestion produces less molecules available for absorption and the following biochemical transformations, resulting in reduced energy required for this work giving a false impression of better efficiency of energy turn-over at low temperatures

(Tirsgaard et al., 2015). Suppressed ADC at the lower thermal tolerance end has been described in other fish species (see Olsen and Ringø, 1998; Satoh et al., 2004; Pace, 2013). This concept may also be applicable for snapper in the present study. As explained earlier (see chapter 2 and 3), snapper and generally sparids are highly sensitive to low temperatures, and particularly is important the impact on metabolic performance at the 12/13°C threshold when snapper start operating less optimally. Similar to Atlantic cod in Trisgaard et al. (2015) the lowest SDA_{coef} observed for snapper was at the lowest experimental temperature (13°C). At that thermal level they were unlikely to be able to exhibit the most economical performance, thus it is suggested that the low ADC at 13°C in snapper is responsible for lowest SDA_{coef} and SDA_{cost} observed.

SDA_{fas}

Factorial aerobic scope of the postprandial peak (SDA_{fas}) as reviewed in Secor (2009), ranges from 1.3 to the extreme of 11 as reported for European eel due to their low SMR. Nevertheless, SDA_{fas} is often only two to three times above the resting baseline level (Jobling, 1993; McCue, 2006; Chabot et al., 2016b) and more importantly in most investigations SDA_{fas} appeared to be independent of acclimation temperatures (Luo and Xie, 2008; Pang et al., 2010; Perez-Casanova et al., 2010; Vanella et al., 2010). However, increase and decrease in SDA_{fas} with rise in temperature was also observed (Pirozzi and Booth, 2009; Frisk et al., 2013). If SDA_{fas} does not change with temperature that means that at any temperature (within the thermal tolerance envelope) it is always the same proportion of energy in a meal distributed between maintenance metabolism and SDA_{cost} (Guinea and Fernandez, 1997). This scenario was observed in *S. aurata* (Guinea and Fernandez., 1997) and to some extent in snapper in the present study, where only a weak positive association with temperature was observed for three experimental thermal levels. Therefore, further investigation at other temperatures is required for a more accurate SDA_{fas}/temperature relationship in *C. auratus* to be revealed.

Furthermore, since SDA_{fas} typically increases with meal size it is not surprising that the peak in both test species, at any temperature measured, did not reach more than two times the resting level. More specifically, snapper SDA_{fas} averaged at 1.5 and YEM 2.0 in association with the predetermined meal size of 0.5% BM ration, which is considered to be at the lower level of ration size commonly selected for SDA work (a meal size range reported in literature extends from 0.25 in Tandler and Beamish (1981) to 24% BM in Fu et al., 2005). Another reason often pointed to as a cause for detecting overall lower SDA_{fas} is a use of routine metabolic rates rather than standard as a baseline level (Secor, 2009; Pirozzi and Booth, 2009), and RMR was a metric of choice for the purpose of this thesis. *S. aurata*, the only other sparid with reported analogous SDA data (Guinea and Fernandez, 1997) to snapper in the present study, exhibited SDA_{fas} with a value of around three at any tested temperatures (i.e. 16, 21 and 26°C). Since most experimental conditions (i.e. ration size, fish size, baseline level and temperature range tested) were comparable between the two studies, only feed type varied (i.e. commercial pellet diet used in Guinea and Fernandez, 1997 versus fish fillets in the present study),

the differences in SDA_{fas} can be at least partially explained by nutritional and calorimetric dissimilarities in the food type used.

Limitation of aerobic scope for activity (SDA_{limit})

When SDA is investigated as a parallel study to determine the growth profile of potential commercial/recreational fish species it is important to quantify how much of the aerobic capacity for activity (i.e. aerobic metabolic scope, AS) is reduced at the point of the SDA_{peak}. In some instances, such as for sit and wait predators, metabolic expenditure during the SDA_{peak} could exceed the level of maximum active metabolic rate (Fu et al., 2005; Wang et al., 2012). Or juvenile Atlantic cod fed with 3.7% BM ration may increase metabolic rate to its maximum levels matching the MO_2 observed for *Ucrit* determination (Soofiani and Hawkins, 1982). Such scenarios potentially leave no room for other aerobic activities with a possibility of impairing maximum growth rates if SDA_{peak} has not been realised. In this study snapper SDA_{limit} was well described as a positive exponential function of temperature where a significant increase between 13 and 17°C was not detected but SDA_{limit} nearly doubled at 21°C. This signified that in snapper, at temperatures close to the highest experienced in the natural environments, between one third and half of AS can be occupied by SDA during its peak after consumption of a relatively small meal. This clearly was not the case for YEM that demonstrated no change in SDA_{limit} between 17 and 21°C. This is another indication that YEM may possess capacity to compensate for temperature change and are able to preserve a higher proportion of AS for other important aerobic activity during the peak SDA response when a relatively small meal is ingested.

4.4.1.2. Effects of ration on SDA response

SDA_{peak}

SDA_{peak} and amplitude in snapper demonstrated a positive linear relationship with meal size. This type of response was also observed for *S. aurata* (Guinea and Fernandez, 1997) and many other fish species (see Soofiani and Hawkins, 1982; Chakraborty et al., 1992; Lucas and Priede, 1992; Ross et al., 1992; Fu et al., 2005; Khan et al., 2015). However, the SDA_{peak}/ration relationship may also be curvilinear since a plateauing tendency with increased meal size has been found in plaice (*Pleuronectes platessa*) (Jobling and Davies, 1980), largemouth bass (*Micropterus salmoides*) (Beamish, 1974), southern catfish (Fu et al., 2005a) and snakehead (*Channa argus*) (Wang et al., 2012). SDA_{peak} in YEM increased with feeding level, but not significantly.

TTP

In contrast to SDA_{peak}, snapper TTP did not show any clear pattern and the only effect due to change in feeding level was between 1 and 2% BM ration. Guinea and Fernandez (1997), however, detected

in *S. aurata* a positive relationship with feeding level, but since they tested effects of ration only for two levels (i.e. 0.74 and 1.47% BM), there is no clear guideline to suggest the TTP response in the present study is typical or due to any differences in experimental protocol and/or subjects used. Nevertheless, snapper TTP data suggest that the time required for the biochemical machinery involved in postprandial work to start running at the highest level does not change at lower rations (i.e. 0.5 and 1% BM). However, since TTP almost doubled between 1 and 2% BM ration, the maximal postabsorptive aerobic response was significantly delayed, corresponding with a surge in high levels of nutrients associated with larger meal. In similar fashion YEM mean TTP increased two-fold when feeding levels increased from 0.5 to 1% BM, yet statistically this was only nearly evident (i.e. $P = 0.056$).

SDA_{dur}

When fish ingest a larger meal usually the time for its digestion is prolonged in comparison to a smaller meal. In fish studies this trend has been frequently reported (e.g. Jobling and Davies, 1980; Chakraborty et al., 1992; Fu et al., 2005a, 2006; Wang et al., 2012). Since fish body mass has been found to have a positive effect on SDA_{dur} (Boyce and Clarke, 1997; Pirozzi and Booth 2009) it appears that the mentioned trend was not effective for YEM SDA_{dur} despite a mean value increase from ~9 to ~13 hours for 0.5 and 1% BM respectively. This is because a significance level was not reached due to the influence of covariate *body mass* on the statistical model, even though a mean body mass difference between two groups was minute (i.e. 30.3 and 33.6 g for 0.5 and 1% BM ratio groups respectively). The aforementioned pattern was also partially supported with snapper data in the current study regardless of a high coefficient of determination ($R^2 = 0.93$) that implied that data were well explained by the linear regression model. However, a closer look at the SDA_{dur}/ration data revealed that the relationship was more sigmoidal or step-wise than linear, since SDA_{dur} was the same for the two lower (0.5 and 1% BM) and for two larger rations (2 and 3% BM) with a two-fold difference between the two ration groups. This pattern, to some degree, resembles TTP where similarly between two smaller and two larger rations TTP did not differ. Therefore, similar processes affected with higher rations that caused the observed TTP pattern may also be responsible for an increase in SDA_{dur}. The trend for SDA_{dur} to increase with meal size was also evident in *S. aurata* but differences were not always significant, which may be related to the sigmoidal relationship with feeding levels observed for *C. auratus* in the present study, if the observed pattern can be extrapolated to other sparids. Nevertheless, Guinea and Fernandez (1997) attributed the lack of significance with ration, that was often present in other studies, to differences in experimental conditions, species, and relatively low ration sizes used (i.e. 0.74 and 1.47% BM). However, in Antarctic Plunderfish, *Harpagifer antarcticus* SDA_{dur} was not affected with ration even though the difference in two meal sizes used was marked (i.e. 2.5% and 14% as satiation ration) (Boyce and Clarke, 1997).

SDA$_{cost}$

It is expected that doubling of meal size would result in doubling SDA$_{cost}$ (Secor, 2009). Along with this notion the cost of digestion was often found to increase linearly with ration size (Vahl and Davenport, 1979; Jobling and Davies, 1980; LeGrow and Beamish, 1986; Lucas and Priede, 1992; Chakraborty et al., 1992; Guinea and Fernandez, 1997, Fu et al., 2005). This was the overall case for snapper in the present study, however SDA$_{cost}$ for the two lowest rations (0.5 and 1% BM) appeared to be independent of feeding levels. Mullet (*C. labrosus* in Flowerdew and Grove, 1980; and YEM in the current study) also demonstrated a positive influence of meal size with doubling the cost of digestion as ration increased from 0.5 to 1% BM. Nonetheless, an exponential increase in SDA$_{cost}$ as a function of increased energy intake has also been demonstrated (Averett, 1968; Tandler and Beamish, 1979).

SDA$_{coef}$

Across fish taxa studied, SDA$_{coef}$ as a function of meal energy has been shown to increase, decrease or remain unchanged. This could be due to species-specific feeding behaviour and adaptive capabilities of their cardio-respiratory system (Fu et al., 2005, 2006), and/or methodological differences in relation to fish activity in the respirometer and variation in nutritional composition of meal (Guinea and Fernandez, 1997). In both snapper and YEM SDA$_{coef}$ did not change with increase in meal size following the most commonly observed trend (Beamish, 1974; Tandler and Beamish, 1980; Ross et al., 1992; Powell et al., 1999, Fu et al., 2005). Nevertheless, snapper SDA$_{coef}$ mean data dropped from ~23 to ~16% between the 0.5 and 1% BM ration, yet not significantly, and remained at the same level for the other meal sizes tested. Interestingly, when data from studies describing a decrease in SDA$_{coef}$ (except of those where only two feeding levels were examined, thus it was not possible to reconstruct actual SDA$_{coef}$/ration relationship) were graphically displayed and an appropriate curve fitted (Fig. 4.9), it was obvious that at lower rations the SDA$_{coef}$ tended to decrease until the lowest level was reached and then continued unchanged as meal size increased. This scenario resembles snapper SDA$_{coef}$ behaviour in the present study (Fig. 4.9). Could it, therefore, be that the described pattern would be also found for other species where a reduction or no change in digestive efficiency was observed if only more feeding levels at higher or lower end respectively were added to the original datasets? Besides, in the current study SDA$_{cost}$ was largely independent of ration size for two lower rations. This was likely responsible for the drop in SDA$_{coef}$ between the two rations (Boyce and Clarke 1997). The same lack of effects of meal size on SDA$_{cost}$ was noted for the few lowest corresponding rations in both Wang et al. (2012) and Fu et al., (2006) further supporting a suggested pattern linking SDA$_{coef}$ and ration size. Therefore, it appears that energetic cost for processing a meal is fixed for the range of feeding levels at the lower end of rations tested but the increase in costs associated with larger meal sizes could be responsible for unchanged SDAcoef at higher rations in some fish species (Boyce and Clarke, 1997; Robertson et al., 2002).

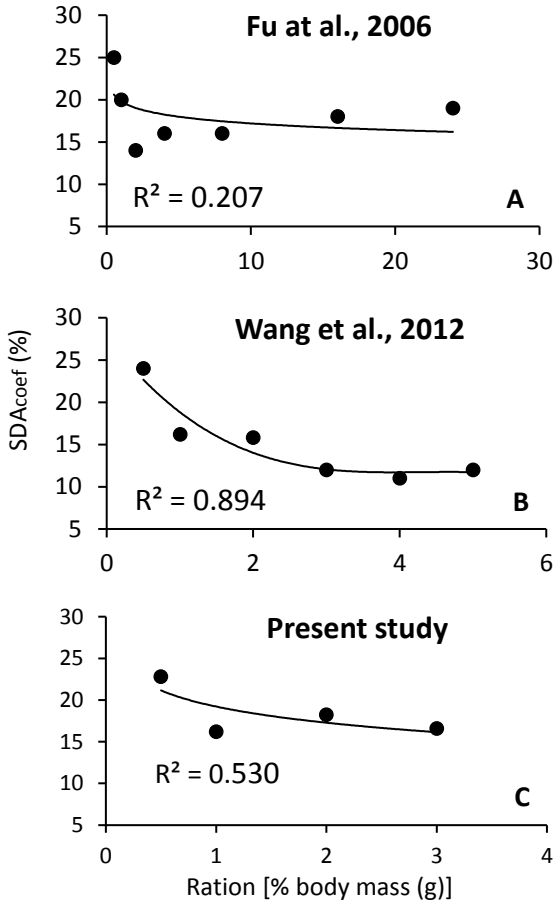

Figure 4.9. SDA coefficient as a function of ration size. A, B and C represent data reconstructed from Fu et al. (2006), Wang et al. (2012), and snapper data from the present study respectively. Black symbols represent means and black curves are models of the best fit with associated R^2.

SDA_{fas}

Jobling (1981b) after reviewing fish SDA studies found that SDA_{peak} continues to rise up to the maximum feeding level that fish would consume but never as high to match the metabolic rate of an actively swimming fish. Hence, he proposed that there is a limit or saturation point to the oxygen demanding processes at the level of cellular metabolism, rather than the lack of capacity of the cardio-respiratory system to deliver oxygen to working tissues, thus not permitting a further increase of SDA_{peak} and SDA_{fas}. SDA_{fas} is often found to increase with meal size where the following two scenarios are most common. First, the scope may continuously increase with ration but the rate of increase is gradually slowing and the plateau is reached before maximum (satiation) ration (Jobling and Davis, 1980, Wang et al., 2012, snapper in this study). And second, the SDA_{fas} continues to increase linearly to the satiation level (Beamish, 1974; Fu et al., 2006). However, less frequently SDA_{fas} is not affected

with ration (Boyce and Clarke, 1997, YEM in this study), which could be possibly because one or more components of cellular metabolism linked to feeding are promptly saturated (Jobling and Davies, 1980; Jobling, 1981b), or too few rations and/or too narrow range of ration sizes was used (Jobling, 1981b). The latter may explain the reason why in the present study YEM SDA_{fas} appeared to be independent of feeding level.

SDA_{limit}

AS was affected by elevated aerobic metabolism during SDA_{peak} and was exponentially related to meal size in snapper, while no effects were observed for YEM. Snapper SDA_{limit} did not change for the first three feeding levels and it markedly increased with the largest ration (3% BM, which can be considered as a satiation/maximum ration for the species) when up to 50% of AS was reduced. Similarly, AS was decreased by 50% at maximum meal size in largemouth bass (*M. salmoides*, Beamish, 1974) and blenny (*Lipophrys pholis*, Vahl and Davenport, 1979). In juvenile Atlantic cod (*G. morhua*) fed with 2.5 and 5% rations estimated reduction of AS was by 40 and 55% (Jordan and Steffensen, 2007). Therefore, it is possible that larger meals could pose higher risk to ecological fitness and survival in some fish due to limitation of fish capacity for other aerobic activities such as locomotion which is crucial for competitive foraging and predator avoidance. It is worth noting that when some other environmental stressor, such as hypoxia, are present during digestion of a large meal, reduction in AS may be even further emphasised. Jordan and Steffensen (2007) demonstrated for Atlantic cod when hypoxia was introduced during 5% BM ration digestion that up to 70% of the scope was taken by SDA_{peak} compared with 50% in normoxia (Jordan and Steffensen, 2007).

4.4.2. Concluding remarks

Amongst three experimental temperatures used to estimate snapper SDA profile at the set 0.5% BM ration, from an ecological perspective it can be suggested that optimal temperature for SDA is likely to be around 17°C. This temperature allows for an moderately rapid meal energy turnover that supports relatively high feeding frequencies. In addition, 17°C is accompanied with low digestion costs with relatively high energy efficiency while at the same time the remaining level of AS (~80%) appears to be sufficient for other important activities. At 21°C, compared with 17°C, due to higher SDA_{peak} and amplitude and shorter SDA_{dur}, growth rates and feeding frequency are expected to be higher. Therefore, temperatures around 21°C may favour commercial interests; however, it comes with higher costs of digestion, reduced energy turnover efficiency and up to 50 % diminished aerobic capacity for activities. When meal size is examined in a parallel manner at 17°C it becomes obvious that 1 % BM ration is probably ecologically and economically the most beneficial meal. SDA_{peak} and amplitude were at the same level as for 2% BM ration but SDA_{dur} was significantly shorter and, similar

to SDA_{cost}, they were both equal with 0.5 % BM ration that yielded better digestive efficiency. Therefore at 1% BM feed intake can be more regular and the meal energy can be incorporated into fish biomass at the same level of efficiency as for larger meals sizes while higher rations (i.e. 2 and 3% BM) appear not to bring any additional benefits in terms of SDA efficiency, but rather less favourable reduction in AS.

Such an elaboration of YEM results was not permissible due to insufficiency of data; however, it can be suggested that temperature may have lesser effects on YEM SDA than meal size, which is consistent with previous findings of this thesis and the fact that YEM are omnivores with a potential preference to eat smaller more frequent meals (Wallace, 1976; McDowall, 1978; Coubrough at al., 2004).

CHAPTER 5

Seasonal growth effects on biochemistry and metabolism of two coastal temperate species

5.1 Introduction

5.1.1 Biochemical growth indicators

Analysing yearly increments deposited in calcified structures, employing bioenergetics models or using tagging/recapture methods are the most common approaches to measuring growth rates in wild fish (Campana and Thorrold, 2001; Gauthier et al., 2008). These help in estimating age and growth rates related to a longer, annual scale. However, estimating growth that would represent shorter time scales as a reflection of current environmental conditions is also relevant since many variables affecting growth oscillate on seasonal, monthly, weekly or even a diurnal basis (Guderley, 2004; Gauthier et al., 2008). In addition, since the biochemical composition of certain tissues could change rapidly with even minor mass alterations, approaches such as condition factors may fail to mirror recent growth rates (Guderley et al., 1996). Relative liver mass (HSI) is regarded as a good representation of growth rates and recent nutritional history in snapper (see chapter 2) and Atlantic cod, *Gadus morhua* (Guderley et al., 1996); however, change in HSI may be more susceptible to shifts in the type of food consumed than to change in growth rates (Black and Love 1986; Jobling, 1988). Therefore, biochemical indices such as activity levels of metabolic enzymes, the concentrations of RNA, the RNA/DNA ratios and the RNA/protein ratios, due to their potential to more closely portray the fish growth performance, have been introduced to supplement and/or substitute morphometric indices (Goolish and Adelman, 1987; Mathers et al., 1992). Concentrations of tissue metabolites as manifestations of important metabolic pathways can further support insights derived from seasonal patterns in activity of key enzymes representing these pathways. The state of energy stores (glycogen, lipids), as well as blood and tissue concentrations of glucose and lactate (the two that can also represent fish activity), are reflections of specific metabolic adaptation to particular seasonal environments where fish with the aid of an 'internal clock' as well as environmental variables can precisely recognise timing of a specific season. Thus, they can rearrange metabolic preferences and

tissue composition in such a way to closely correlate with the rhythm of seasonal environmental change (Koch et al., 1992; Guderley, 2004; van Dijk et al., 2005).

5.1.1.1 Overview of key enzymes in major metabolic pathways important for growth and energy homeostasis in fish

Pyruvate kinase (PK)

In fish as in any other organisms, glycolysis is the only way for a molecule of glucose to be catabolised (Cowey and Walton, 1989). An entire array of enzymes required to produce two molecules of pyruvate from one molecule of glucose have been identified in fish (Walton and Cowey, 1982) including PK as a terminal catalyst in the chain reactions responsible for turning phosphoenolpyruvate into pyruvate. Because of its position in the pathway PK is numbered amongst the rate-controlling enzymes of glycolysis, together with hexokinase and phosphofructokinase (Enes et al., 2009). In the presence of oxygen, a molecule of pyruvate can be transported into mitochondria and via pyruvate dehydrogenase oxidised into acetyl CoA and as such enters the Krebs (citric acid) cycle resulting in the production of one molecule of ATP (adenosine triphosphate, the main energy compound in living cells). More importantly the Krebs cycle is responsible for reduction of the coenzymes nicotinamide adenine dinucleotide (NAD+) and flavin adenine dinucleotide (FADH) to NADH and FADH2 as universal electron carriers/acceptors in the electron transport chain (ETC) which terminates with ATP production as the final step in the oxidative phosphorylation.

Lactate dehydrogenase (LDH)

In anaerobic conditions ETC and oxidative phosphorylation are unable to perform due to the lack of the terminal electron acceptor, oxygen, needed for the proton gradient to be established, therefore mitochondrial ATP production is compromised (Garrett and Grisham, 2005). Under these circumstances the only source of ATP is via anaerobic glycolysis, and even though this approach is inferior to mitochondrial energy generation (i.e. 2 versus 36 molecules of ATP produced in anaerobic glycolysis and in mitochondrial respiration respectively), in white muscle it is a vital energy production pathway (Moyes et al., 1989). The terminal product of anaerobic glycolysis is lactate which is reduced from pyruvate by the catalytic activity of LDH, the enzyme recognised as a rate setting regulator of the respective pathway (De Silva and Anderson, 1995). LDH is a bisubstrate enzyme, also utilising lactate as a substrate performing reversible reaction to oxidise lactate back to pyruvate from where it could take several routes such as conversion back to glucose via gluconeogenesis or through a reaction with acetyl-CoA to fatty acids (Berg et al., 2012).

Citrate synthase (CS)

Aerobic metabolic activity is often portrayed by the markers of mitochondrial abundance, enzymes CS and cytochrome *c* oxidase (CCO), representatives of the Krebs cycle and electron transport chain respectively (Thibaoult et al., 1997; Morbey et al., 2010). CS is involved with an initial step in the citric acid cycle, where acetyl-CoA, as one of the catabolic end products of fats, carbohydrates and proteins, via the condensation reaction with oxaloacetate forms six carbon citrate (Wiegand and Remington, 1986). CS is considered as the rate-limiting enzyme of the Krebs cycle (Alp et al., 1976; Somero and Childress, 1985; Gauthier et al., 2008); however, Cooney et al. (1981) argued that this may not be the case since they demonstrated that the capacity of the Krebs cycle of a rat heart could be augmented by increasing levels of α-ketoglutarate dehydrogenase (the fifth reaction in the cycle) without changing the levels of CS. Nevertheless, activity of CS has been found to positively correlate with the capacity of the Krebs cycle (Guderley and Gawlicka ,1992), thus it has been often utilised as a useful indicator of tissue aerobic potential (e.g. Goolish and Adelman, 1987; Pelletier et al., 1993; Blier et al,. 1997; Couture et al., 1998; Dutil et al., 1998).

CCO

ETC is composed of four complexes and three proton pumps, with an overall function to generate the proton gradient necessary for the final step in oxidative phosphorylation – the production of ATP performed by ATP synthase (Berg et al., 2012). The terminal complex and proton pump in the chain is represented by CCO. Unlike the aforementioned controversy about rate determining properties of CS in citric cycle, CCO is universally agreed to perform a function that controls the rate of ATP production in mitochondria (Groen et al., 1982; Tager et al., 1983; Heineman and Balaban, 1990; Pelletier et al., 1993; Blier and Lemieux, 2001). In support of this notion Villani et al. (1998) demonstrated that the maximal activity of human cells' CCO measured *in vivo* could be almost identical to the rate of electron transfer, thus CCO can be considered as the key indicator of aerobic ATP synthesis (Goolish and Adelman, 1987).

3-hydroxyacyl CoA dehydrogenase (HOAD)

Beta oxidation of fatty acids is coupled with both the Krebs cycle and the respiratory chain; therefore, it is regarded as a principal mitochondrial pathway responsible for energy turnover from the lipid source (Tyni et al., 1999). Beta oxidation is an important energy pathway even when food is not limited, which is especially true for fish red muscle and heart (except for the elasmobranch myocardium, which is unable to catabolise fatty acids) where lipids, together with carbohydrates and ketone bodies are a continuous source of ATP production (Sidell et al., 1987; Moyes et al., 1989). Four enzymatic reactions make the repeated cycle of beta oxidation pathway possible. Every cycle ends with the shortening of fatty acyl-CoA by two carbon atoms and production of acetyl-CoA, with its fate in the Krebs cycle or conversion into ketones, and reduced coenzymes NADH, and $FADH_2$ predetermined for utilisation in ETC. A molecule of water and five ATP are also produced during each

beta-oxidative cycle, and the process continues until the entire fatty acyl-CoA molecule has been reduced to a set of acetyl-CoA molecules. HOAD is a catalyst of the penultimate reaction in the mitochondrial fatty acid oxidation spiral, where 3-hydroxyacyl-CoA is converted to 3-ketoacyl-CoA and a molecule of NAD+ takes an electron to be reduced into NADH (Clayton et al., 2001).

5.1.1.2 Overview of key enzymes of protein digestion in fish

Trypsin and Chymotrypsin

Digestion as an integral process of animal physiology relies principally on the catalytic properties of digestive enzymes, the functions of which have been well described in many vertebrates including fish (Infante and Cahu, 2001). Organs such as stomach, exocrine pancreas and intestine are the primary centres for digestive enzymatic production (Berg et al., 2012). Activity of acidic proteases is largely found in the stomach where the zymogen pepsinogen is activated when in contact with hydrochloric acid and becomes a principal protease pepsin. The most potent digestive gland, the exocrine pancreas oversees production and secretion of glucosidases, lipases and proteases, an array of enzymes covering digestion of all three types of macronutrients (Infante and Cahu, 2001). Those proteases operate at alkaline pH after activation by trypsin, which in turn is transfigured from its pro-enzyme form trypsinogen by a transmembrane serine protease, enterokinase (enteropeptidase) produced by epithelial cells (enterocytes) of the small intestine (Berg et al., 2012). The two most important proteolytic enzymes in the alkaline digestive system are serine (they possess a serine residue in their catalytic site) endoproteases, trypsin and chymotrypsin (Rungruangsak-Torrissen and Male, 2000; Simpson, 2000). Even though trypsin is a main enzyme stimulating zymogens (including chymotrypsinogen) in the intestinal lumen, it has a relatively narrow specificity for peptide bonds, performing hydrolysis only at the carboxyl side of arginine and lysine, while chymotrypsin possesses a much larger range of specificity, cleaving peptide bonds containing amino acids with large side chains and nonpolar amino acids including tyrosine, phenylalanine, tryptophan, and leucine (Simpson, 2000). In terms of the actual production site of serine proteases, there is no uniformity in the realm of fish since a distinct pancreas is not always present, but rather pancreatic exocrine acinar cells are often found in the pyloric caeca, finger–like pouches located posterior to the stomach (Kapoor et al., 1976; Ring et al., 2003). In many other cases the pancreatic like tissues are dispersed around the upper intestines or are incorporated as islets within the liver in which case the formation is termed hepatopancreas (Geyer et al., 1996; Kozarić, 2001; Petcoff et al., 2006; El-bakary and El-gammal, 2010; Nejedli and Gajger, 2013).

5.1.1.3 Enzymatic activity and growth performance in fish

The relationship that links environmental conditions (i.e. food availability) and activities of certain enzymes of energy metabolism have been demonstrated for many fish species, including rainbow trout (*Oncorhynchus mykiss*) (Nikki et al., 2004; Churova et al., 2010); Atlantic salmon (*Salmo salar*) (Hevroy et al., 2006); coho salmon (*Oncorhynchus kisutch*) (Hill et al., 2000); Atlantic cod (Pelletier et al., 1995; Couture et al., 1998; Dutil et al., 1998;); carp (*Cyprinus carpio*) (Ennion et al., 1995); lake trout (*Salvelinus namaycush*) (Morbey et al., 2010); spotted wolfish (*Anarhichas minor*) (Imsland et al., 2006); snapper (*C. auratus*) (Majed et al., 2002b) and others. In addition, since there is a clear relationship between ingested food and growth rates (Houlihan, 1991), it is expected that there will be an association between activities of those enzymes and the rate of fish growth (Pelletier et al., 1995). In support of this assumption, a strong positive correlation between the activity of the glycolytic enzymes PK, PFK and LDH from Atlantic cod (*Gadus morhua*) white muscle and their growth rate has been demonstrated (Pelletier et al., 1993a). Similarly, in Atlantic cod and saithe (*Pollachius virens*), growth has been observed to mirror the activity of aerobic mitochondrial enzymes CCO and CS from the same white muscle tissue (Mathers et al., 1992; Foster et al., 1993; Pelletier et al., 1993b). Furthermore, other studies have indicated that besides white muscle, the digestive tract may also be a target tissue to search for biochemical correlates with growth, especially CCO and CS in the intestine, as the link between their activity and growth rates has been demonstrated (Blier et al., 1997; Couture et al., 1998; Dutil et al., 1998). However, it has been frequently observed that activity of mitochondrial enzymes is not as reliable a growth rate indicator, at least for some species (e.g. cod), as glycolytic enzymes that exhibit a more consistent relationship with growth (Pelletier et al., 1995).

The importance of beta oxidation for growth, represented through the activity of HOAD, has been studied in relation to the transition in utilisation of different substrates for energy generation (carbohydrates/lipids) in two fish muscle types (white and red) during ontogenetic development (Kiessling, 1991). As muscle tissue is ontogenetically differentiated into fully functional white and red muscle it was followed with an increase (RM) and decrease (WM) of HOAD activity (Kiessling, 1991). Its activity measurements also accompanied several studies exploring changes in metabolic organisation of different tissues affected by change in growth rates (Pelletier et al., 1994), and as a function of seasons, where the highest activity was observed in winter and lowest in autumn suggesting increased reliance on fat reserves in Atlantic cod WM during the coldest months in opposition to energy accumulation in autumn (Pelletier et al., 1993).

Serine proteases have also been found to complement growth studies where they often demonstrated their potential as growth indicators. Trypsin activity has been shown in several species (e.g. Atlantic salmon and cod, Torrisen and Shearer, 1992; Lemieux et al., 1999 respectively) to be

linked to growth rate and food conversion efficiency. Furthermore, trypsin was found to be an indicator for nutritional condition and digestive capacity for early life stages in herring (*Clupea harengus*) (Ueberschaer et al., 1992) and sea bass (*Dicentrarchus labrax*) (Cara et al., 2007), and a specific trypsin allozyme has been associated with better protein utilisation and faster growth in Atlantic salmon and Arctic charr (*Salvelinus alpinus*) (Torrissen, 1991; Torrissen et al., 1994). In addition, Rungruangsak-Torrissen et al. (2006) demonstrated that not only trypsin but also trypsin/chymotrypsin (T/C) activity ratio can be a useful tool for growth studies either when cultured fish or wild populations are concerned under conditions when rate of feed intake cannot be measured. They showed that trypsin activity and T/C ratio increased in Atlantic salmon parallel with increased growth and that the ratio dropped during starvation.

5.1.1.4 Enzymatic activity and seasonal effects in fish

Fish are equipped with sensory systems to monitor the rate and direction of change in environmental cues (e.g. temperature and photoperiod) to "predict" forthcoming cold or warm conditions and to adjust their behavioural and/or metabolic responses to a seasonal event in a way that would best suit their lifestyle (Egginton et al., 2000; Guderley et al., 2001; van Dijk et al., 2005). There are three general approaches to how fish deal with the predictable change in seasonal temperatures – fish can slow down metabolic rates congruently with a decrease in temperature and submit to Q_{10} effects; or as winter approaches they can enter into dormancy below a threshold temperature; finally they can offset the Q_{10} effects by using single or an array of strategies to partially or perfectly compensate for the reduction in metabolic capacity that may result from a drop in temperature (Thibaoult et al., 1997; Guderley, 2004).

The trend commonly observed in temperate fish that tend to stay active during the coldest season is to quantitatively and/or qualitatively increase the aerobic capacity of a tissue (St-Pierre et al., 1998; Guderley, 2004). Quantitative modification due to cold acclimation may involve an increase in the proportion of oxidative fibres in muscle tissue and increase in mitochondrial abundance or mitochondrial volume density; whereas qualitative modifications include altering the activities of mitochondrial enzymes, an increase in mitochondrial cristae density and alteration of mitochondrial phospholipid membrane properties (Wodtke, 1981; Johnston, 1993; Sänger, 1993; Guderley, 2004). Fish adapted to cold environments may have higher mitochondrial mass per wet gram tissue than those from warm habitats, which in terms of oxidative capacity is observed with 2–5 times more CCO and CS activities at 1°C in Antarctic compared with temperate fish taxa (Crockett and Sidell, 1990). Primarily in oxidative (red), but also in glycolytic (white) muscle of moderately active eurythermal/temperate species a negative relationship is often reported between mitochondrial

volume density and habitat temperature (Johnston et al., 1998), since mitochondria are in general the main site/target of thermal compensation (Guderley, 2004). One of the most commonly observed alterations in mitochondria to cold is adjustment of membrane phospholipid and fatty acid composition to maintain membrane function at lower temperatures (Hazel and Landry, 1988; Guderley, 2004). This modification is often coupled with increased capacity for lipid oxidation (i.e. accompanied by increased HOAD activity) and storage, such as has been observed for rainbow trout and striped bass (Jones and Sidell 1982; St-Pierre et al., 1998; Egginton et al., 2000). This increased lipid metabolism and change in lipid membrane composition enhances a flux of oxygen to additionally support aerobic metabolism in the muscle tissue, since oxygen dissolves easier in lipids than in water (Egginton and Sidell, 1989; Desaulniers et al., 1996). However, in many instances, an effort to maintain aerobic capacity when facing cold, in some temperate species is not found (Bremer and Moyes, 2011). This observation is not necessarily referring only to phylogenetically diverse taxa but also to closely related species with a similar locomotory and life style patterns (for the list of examples see Bremer and Moyes, 2011). Further, the response to cold can be in contrast within the same species between studies as has been demonstrated for rainbow trout (cf. Thibaoult et al., 1997; St-Pierre et al., 1998). The opposing findings were assigned to a difference in fish condition, strains, and the type of analytical approach (St-Pierre et al., 1998).

Activities of glycolytic enzymes have not been often demonstrated to distinctly vary with seasons, therefore they are generally considered to be relatively stable in the face of cold acclimation (Sidell, 1983). Nevertheless, exemptions have been reported (Blier and Guderley, 1988), since activities of LDH, PK and phosphofructokinase (PFK) for some fish have been found to be sensitive to change in ambient temperature (Shimero et al., 1997; Couture et al., 1998; Rennie et al., 2005; Kaufman et al., 2006; Gauthier et al., 2008). More specifically, spring has been associated with increases in glycolytic activities in salmonids, when augmented anaerobic activity is required primarily for prey capture as their abundance increases with rise in temperature after cold winter period (Childress and Somero, 1990; Garenc et al., 1999; Martinez et al., 2003; Morbey et al., 2010). On the other hand, an increase in glycolytic enzymes can also be considered as sparing protein and fat stores (Houlihan et al., 1988).
The trend in digestive enzymes, in terms of seasonal fluctuation, at least for salmonids, shows that the activity decreases in winter possibly as a response to decreased food intake and reduced appetite and it is kept at low levels until spring when the activity rapidly rises with the peak in summer again associated with increased foraging activity and amount of food ingested (Einarsson et al., 1997). However, in some other temperate species, such as rudd (*Scardinius erythrophthalmus*) proteolytic activity was found to change only little throughout the annual cycle (Hofer, 1979).

5.1.1.5 Main tissue metabolites, overview of metabolism in fish

Important information to complement the understanding of dominant metabolic pathways can be achieved by measuring tissue concentrations of low-molecular-weight compounds as indicators of specific metabolic activity (lactate and glucose) and energy stores (glycogen).

Lactate metabolism in fish

Lactate or its protonated form, lactic acid, is a dynamic intermediary metabolite of carbohydrate metabolism (Omlin et al., 2014). Contractions of white muscle (WM) are engaged when a need for burst-type movement is required (e.g. catch prey, evade a predator, confront strong currents). Due to its low aerobic capacity, likely because of constraints in oxygen supply and mitochondrial density, WM is not able to aerobically satisfy energetic needs for such activities (Hochachka, 1987). Hence anaerobic metabolism via phosphagen hydrolysis and anaerobic glycolysis is employed (Moyes et al., 1989). Pyruvate, as an end product of glycolysis, in the absence of oxygen is reduced to lactate by LDH. Simultaneously NADH is oxidised (i.e. lose an electron) to NAD+, which is important for continuous glucose catabolism and cellular redox balance. Previously it was thought that accumulated muscle lactate was a nuisance waste product likely causing muscular acidosis and fatigue with the resulting cytological dysfunctions (Blier et al., 1997; Chatham, 2002). In recent decades this paradigm has radically changed (Nalbandian and Takeda, 2016). Firstly, lactate was excluded as a plausible candidate responsible for post exercise cellular pH decrease, since all the intermediate acids of glycolysis, including lactic, have low pK (i.e. acid dissociation constant) forcing them to exist in base form. Therefore, the H+ production, liable for the acidosis, cannot originate from lactic acid but more likely from ATP hydrolysis (for a review see Robergs et al., 2004). Secondly, lactate has been recognised as an important energy source and the preferable fuel for the oxidative tissues such as heart (Lanctin et al., 1980; Milligan and Farrell, 1991; Chatham, 2002) and brain (Soengas and Aldegunde, 2002), and generally it is seen as both an instantaneous energy source and an important precursor for rebuilding energy reserves (Enes et al., 2009; Nalbandian and Takeda, 2016). Red muscle (RM) is predominantly powered by mitochondrial oxidation of fatty acids and ketone bodies, but also by pyruvate that may originate from glucose, glycogen or lactate (Moyes at al., 1989). Unlike RM, WM seems to rely mostly on anaerobic pathways (Crabtree and Newsholme, 1972; Kieffer, 2000); however, its basal metabolic requirements appear to be met aerobically (Wardlaw and Kaplan, 1984). It has been argued that mitochondrial oxidation of lactate, glucose, ketone bodies, and fatty acids saves glycogen stores and restocks phosphagens as a main fuel required for anaerobic, fervent WM work (Moyes et al., 1989). Gluconeogenesis is a metabolic route that results in the generation of glucose by utilisation of non-glycosidic substrates including lactate, glycerol, *a*-keto acids and certain amino acids (Hemre et al., 2002; Enes et al., 2009). Conservative views on the matter of lactate as a substrate for gluconeogenesis are often explained from a mammalian perspective by the Cori cycle which describes

the shuttle of lactate from muscle to liver where it is oxidised to pyruvate and via a reversing pathway converted into glucose. Glucose is subsequently transported back to muscle to be catabolised or utilised for glycogen restoration (Wood, 1991). The importance of this principle has been challenged for higher vertebrates (Johnson and Bagby, 1988; Hoshino et al., 2014) and even more for fish where there is a great body of evidence supporting the idea that most (80–85%) of the lactate produced by WM remains intramuscularly and is essentially metabolised *in situ* primarily for replenishment of depleted glycogen stores (Wood, 1991; Milligan and Girard, 1993; Milligan, 1996; Gleeson, 1996). Moreover, WM tissue actively takes up circulating lactate, and it has been demonstrated by a hepatectomy (the surgical resection/removal of the liver) in rainbow trout that the WM glycogen recovery was augmented suggesting that uptake of lactate by other organs and tissues, especially post-exercise negatively affects the rate by which WM can metabolically recover (Milligan and Girard, 1993). Omlin et al. (2014) went even further and suggested that lactate production is an essential requirement for trout and is needed for oxidative tissues and/or for those relying on glycolysis. In addition, lactate conversion back to pyruvate is regarded as a vital mechanism to regulate the redox balance in cells, and its function as an intracellular signalling molecule for several anabolic pathways and as a brain plasticity regulator has been also recognised (Philp et al., 2005; Mosienko et al., 2015; Nalbandian and Takeda, 2016).

Most of fish lactate production is linked to WM burst swimming action. Requirements for such an activity reflects changes in seasonal environmental conditions such as seasonal change in temperature, food availability, predator pressure and spawning activity. Therefore, tissue lactate content can be considered as an indicator of seasonal effects on fish behaviour and physiology, but this would depend on relatively stable levels of lactate.

Glucose metabolism in fish

Glucose seems to be an imperative energy source for particular tissues in fish such as the brain, gills, erythrocytes, kidneys and gonads (Tseng and Hwang, 2008; Polakof et al., 2012), as well as white muscle via its stored form glycogen that together with ATP and creatine phosphate fuel burst-type activity (Kieffer, 2000). In normoxic conditions in oxidative tissues glucose is entirely catabolised/oxidised through glycolysis – Krebs cycle – ETC for ATP synthesis. However, after glucose starts to be catabolised via glycolysis, its first product glucose 6-phosphate may also enter the anabolic pathway known as the pentose phosphate shunt that finalises with the production of cytosolic NADPH and ribose 5-phosphate, which are directed towards lipid and nucleotide biosynthesis respectively (Hemre et al., 2002; Enes et al., 2009). Excess glucose, depending on specific tissue requirements and preferable metabolic pathways, may be deposited as glycogen via glycogenesis or enter lipogenesis to be converted to lipids (Polakof et al., 2012). Starvation normally induces utilisation of stored or *de novo* glucose produced through gluconeogenesis. Therefore, glucose homeostasis (i.e. balance

between glucose production and storage) is maintained by nutritional and hormonal means by regulating key enzymes associated with those pathways (Pilkis and Granner, 1992).

Even though it has been recognised as crucial for certain tissues and organs, some authors argue that glucose metabolism in fish, except for tunas and eels, may not be overall as important as metabolism of proteins and lipids (Hemre et al., 2002), or as important as for higher vertebrates (Wilson, 1994; Hemre et al., 2002; Stone, 2003). There are many reasons for this suggestion. First, many fish species are considered glucose intolerant to dietary or intravenous glucose load, displaying markedly longer hyperglycaemia (above normal levels of blood glucose) relative to higher vertebrates. However, omnivorous and herbivorous fish taxa reach normoglycemia quicker, possibly because they possess higher densities of insulin receptors than carnivorous fish (Legate et al., 2001; Moon, 2001). Nevertheless, compared with mammals, blood insulin in fish generally seems to be at similar levels. Therefore, the reason for prolonged hyperglycaemia may not be related to plasma insulin levels but possibly to low peripheral utilisation of glucose as a reflection of fewer muscle insulin receptors (Navarro et al., 1999) and/or differences in glucose transport and cytosolic hexokinase/glucokinase activity (Legate et al., 2001; Moon, 2001). It is worth noting here that the function of insulin may not be entirely the same as in mammals and even though its role in regulating plasma glucose has been described (Moon, 2001), it appears that it is more closely related to growth and amino acid metabolism (Mommsen and Plisetskaya, 1991). In addition, since there is a relatively high level of muscle glucose production and utilisation but relatively small metabolic exchange with the rest of the body, glucose and glycogen turnover appears to be generally a closed activity/system within the muscle tissue itself (Milligan, 1996; Hemre et al., 2002). Furthermore, carbohydrate from the diet has been shown to be linked to lipid metabolism as dietary sugar often ends in the pentose phosphate pathway, which is important for the increased availability of the cytosolic reducing potential in the form of NADPH needed for fatty acid biosynthesis (Hemre et al., 2002). Since this process is highly detectible in fish liver, it is believed that liver is the primary organ for fish lipogenesis which may be controlled by liver carbohydrates (Hemre et al., 2002).

Even though glucose is marginalised in metabolic importance in fish relative to mammals, this is definitely not the case when it comes to the energy supply for iono- and osmoregulation tissue and organs, where carbohydrates are the primary energy source (Tseng and Hwang, 2008).

Glycogen metabolism in fish

Glycogen stored in muscle cannot be regarded as a general energy emergency pool due to its closed role in muscle performance (Hemre et al., 2002). Hence liver, despite containing smaller amounts relative to muscle mass, can be considered as a main glycogen store in fish (Soengas et al., 1996). However, there is no universal response to food deprivation in terms of utilisation of liver glycogen, similarly hepatic glycogen content may markedly vary in fish (i.e. 1–12% of liver wet mass), therefore

it may be of a different importance as an energy store to different fish (Hemre et al., 2002; Enes et al., 2009). Gilthead sea bream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*), rainbow and brown trout (*Salmo trutta*) exhibit distinct depletion of hepatic glycogen content as a result of starvation (Navarro and Gutierrez, 1995; Meton et al., 2003; Soengas et al., 2006; Perez-Jimenez et al., 2007), while American eel (*Anguilla rostrata*), common carp (*Cyprinus carpio*) and Atlantic salmon do not mobilise liver glycogen when fasting but rather depend on lipid stores (Nagai and Ikeda, 1971; Moon, 1983; Sundby et al., 1991). Two enzymes, glycogen synthase and glycogen phosphorylase, are responsible for glycogenesis and glycogenolysis respectively. Control of their activity is hormonal, and it is manifested through phosphorylation and dephosphorylation reactions. Increase in the glucagon/insulin ratio stimulates phosphorylation, which promotes activation of glycogen phosphorylase causing increased glycogen breakdown, and deactivation of glycogen synthase which is responsible for cessation of glycogen synthesis, and the opposite applies with a decrease in glucagon/insulin ratio (Enes et al., 2009). In many temperate fish species, a winter period is often associated with both decline in food availability and decrease in feed intake. Therefore, winter fasting can be monitored through depletion of glycogen stores in some fish, giving another metric that can be utilised for detection of seasonal effects on fish growth and condition.

5.1.2 Snapper and YEM in biochemical research

When it comes to snapper and YEM, there is a literature scarcity in respect to work on biochemical markers for growth. However, the seasonal variation in activity of enzymes of anaerobic glycolysis represented by LDH and aerobic metabolism by CS in snapper was examined by Majed et al. (2002b). They observed that both enzymes correlated with snapper growth in length, where the LDH increase was explained in terms of meeting the requirement for swift anaerobic activity; while CS, which followed the seasonal somatic growth cycle, reduced its presence in the muscle as body length increased, which possibly was due to a reduction in energy generation (Majed et al., 2002b).

On the other hand, measurements of tissue metabolites have been obtained in several investigations (Black, 2002; Black et al., 2004; Cook et al., 2012; Tukey et al., 2012 and Coxon, 2014), but very few data reflected growth and/or seasonally related effects. This was the case only in Black (2002) where WM lactate from rested YEM was measured in winter and summer but the purpose was not to examine seasonal impact on the content but rather the focus was on white muscle cell viability during post-mortem storage.

5.1.3 Objectives of the Chapter

When determination of a biochemical profile with the purpose to broaden the understanding of fish growth in a seasonal environment are considered, traits of aerobic and anaerobic metabolic pathways, as well as digestive capacity, may be investigated. Knowledge of activities of key enzymes associated with those pathways, together with tissue metabolites as associated metrics, all of which have been shown to be linked to growth, can complement morphometric and respirometry findings and as such assist in future production enhancements.

Therefore, objectives of this chapter sought to assess the biochemical state of the test species by determining annual activity dynamics of enzymes with the potential to correlate with growth performance and which are important catalysts in main biochemical pathways (i.e. cytochrome *c* oxidase, citrate synthase, pyruvate kinase, lactate dehydrogenase, 3-hydroxyacyl-CoA dehydrogenase, trypsin and chymotrypsin). Their activity dynamics were subsequently compared with growth morphometrics (chapter 2).

Note that in liver, only HOAD activity was determined. The reason for this was because intriguing dynamics of visceral fat deposition was observed in both test species that brought the question about liver participation in the process of lipid creation and deposition. If liver would have been involved in the process, this would be reflected in annual trajectory of HOAD activity in the organ.

Another aim, concurrently with enzymes, was to determine concentrations of key tissue metabolites – lactate, glucose and the energy store compound glycogen, which further aided in evaluation of metabolic pathways involved during the growth of test fish through the period of 12 months on controlled (unrestricted) diet under environmental conditions.

5.2 Material and Methods

5.2.1 Experimental animals

Experimental animals originated from the same source as described in the methods section of chapter 2. This methods section is a continuation of the procedures depicted in section 2.2 of this thesis. The final step listed in 2.2.3.3, on a day when a sampling/measurement session was taking place, was to select eight (for all YEM sessions and the first six snapper sessions) or seven (the last five snapper sessions) fish from the tank for subsequent euthanasia and laboratory work. The laboratory work on the sampling day encompassed: euthanasia, dissection, organ and fat deposit weights, tissue sampling, snap-freezing and transfer of frozen samples to a -80°C freezer for following biochemical analysis.

5.2.2 Euthanasia, dissection and morphometric data acquisition and preparation of samples for biochemical analysis

Fish euthanasia was carried out immediately after all fish in a tank were processed (i.e. measured and moved) during a sampling/measurement session. The method of euthanasia practiced was the *iki-jime* technique, which is abrupt brain ablation with a spike inserted directly into the fish hind brain. The aim was first to locate a small depression in the fish cranium (skull) just above and behind the fish eye. Penetration with a sharp instrument through the depression in the medio-caudal direction leads directly to the hind brain, which is instantly destroyed with a few circular motions. When the hind brain is hit the fish responds with a short and strong convulsion before it dies. The *iki-jime* technique was considered to be the most ethical method for fish euthanasia due to its efficiency and swiftness.

After euthanasia each fish was photographed for the PFR database, weighed on the SB8001 Precision Balance (Mettler-Toledo, LLC, Columbus, OH 43240, resolution to 0.1 g) and length measured in the same way as for fish in the growth study earlier in the day. Fish were placed in numerically labelled zip plastic bags and put in a 40 L crate filled with sea water and ice-block bags. Fish were dissected starting with opening the abdominal cavity with blunt pointed scissors. First the fish heart was extracted out of the pericardial cavity which enabled easy access to the oesophagus. The oesophagus was severed and this enabled the entire internal organs to be removed with a gentle pull. During the excavation of visceral mass, extra care was devoted to cleaning the abdominal cavity of all fat deposits and other tissue remnants and then the gutted mass of the carcass was weighed and

recorded. Next, the fish liver was traced and the gallbladder carefully removed, which allowed the liver to be detached. Then the spleen was found and separated from the visceral material. Following this, the stomach with oesophagus was severed from the pyloric caeca connected to the intestines and everything was thoroughly cleaned of fat deposits. Once all organs (i.e. heart, liver, spleen and pyloric caeca with intestines) were separated and the entire visceral fat gathered they were individually weighed on an AB104-S Analytical Balance (Mettler-Toledo, LLC, Columbus, OH 43240, USA, precision: 0.1 mg, resolution: 0.1 mg) and mass values were recorded. After weighing, preparation of tissue samples followed. A sample of 200–300 mg of liver was separated into two samples, one for enzyme activity and another for metabolite determination. The pyloric caeca were disconnected from the intestines forming two samples each for different enzymatic activity determination. White muscle (WM) samples were obtained by incision of the anterior part of the dorsal muscle section just above the lateral line that was vertically aligned with the pectoral fin, as described in Jerrett et al. (1996) and Jerrett and Holland (1998). WM sample block was cubed into three parts each weighing approximately 250 mg. The red muscle (RM) samples were incised from the skinned fillets, as after the skinning the narrow (Snapper) or wider (YEM) strip of RM was revealed alongside the middle of the fillet. RM was carefully trimmed of attached WM and fats and divided in two separate samples from ~50 mg (~30 g Snapper) – 150 mg (> 50 g Snapper and all YEM). Once all samples were acquired they were placed on labelled aluminium foil squares, folded up and put into a container of liquid nitrogen. The samples were subsequently moved to a -80°C freezer to be stored until scheduled for the biochemical analysis.

5.2.3 Tissue homogenisation for enzyme extractions and metabolites quantification

All procedures were the same for both test species unless stated otherwise.

Seven different enzymes and three types of tissue metabolites from different organs as outlined in 5.1.3 were of interest in this study. Enzyme extractions were carried out according to protocols derived from preliminary trials that were based on comparable published work.

5.2.3.1 Homogenisation protocols for generation of enzyme extraction samples

The first step in enzyme extraction from selected samples started with the homogenisation process as described below.

Three to four batches of 14 (i.e. five last sampling points for snapper) or 16 (i.e. all other sampling points) tissue samples were homogenised on the same day. Whenever feasible the entire set of

samples belonging to a specific tissue type (i.e. derived from all sampling points) were processed within the same week or less. Full attention was dedicated to ensure that the conditions of homogenisation were identical between days, which included the use of freshly made chemicals, working in a constant room temperature of 20°C and following the same protocol for the tissue type. The same principle was applied for tissue metabolites determination.

Most of the chemicals used for homogenisation and enzymatic activity determination were sourced from Sigma – Aldrich (St. Louis, MO, USA); others, like calcium chloride dihydrate ($CaCl_2$ X $2H_2O$), potassium chloride (KCl) and Triton X-100 ($C_{34}H_{62}O_{11}$) were obtained from Thermo Fisher Scientific New Zealand Ltd (Albany, Auckland, New Zealand); and EDTA (ethylenediamine-tetraacetic acid, $C_{10}H_{14}N_2Na_2O_8$*$2H_2O$) and magnesium chloride-6-hydrate ($MgCl_2$*$6H_2O$) from Scharlau- Scharlab, S.L. (Barcelona, Spain).

5.2.3.1.1 WM enzyme extraction protocol

In WM, activities of the following enzymes were determined:

Cytochrome *c* oxidase (CCO) (EC 1.9.3.1)
Citrate synthase (CS) (E.C. 4.1.3.7.),
Pyruvate kinase (PK) (EC 2.7.1.40).
Lactate dehydrogenase (LDH) (EC 1.1.1.27)
3-hydroxyacyl-CoA dehydrogenase (HOAD) (EC 1.1.1.36).

Stages of enzyme extraction:

1. WM tissue samples were taken out of -80°C storage and kept at -20°C during the homogenisation process. First, a WM block sample while still frozen, was cleaned of any blood stains and fragments of red/pink muscle, before it was thinly sliced with a scalpel blade and minced well to form a finely mashed material of approximately 100 mg.

2. The mashed muscle was put in a labelled 2 ml snap-top eppendorf tube (Eppendorf® Safe-Lock®, Eppendorf AG, Hamburg, Germany) and weighed. The eppendorf tube was placed on the eppendorf cooling block (at 0°C) until the entire batch was ready for the next step. Between two preparations the surface of the plastic chopping board was cleansed with 100% ethanol.

3. Into each tube 300 µL ice-cold homogenisation buffer was pipetted with the Eppendorf Multipette Xstream Repeater (Eppendorf AG, Hamburg, Germany). Homogenisation buffer (pH 7.5) was prepared from the following reagents using nano-pure water (~18 MΩ x cm):
   - 20 mM HEPES (4-(2- hydroxyethyl) piperazine-1-ethanesulfonic acid)
   - 1 mM ethylenediamine-tetraacetic acid (EDTA)
   - 2 mM $MgCl_2$ (magnesium chloride)
   - 0.1% Triton X-100

4. A scoop (~150 µL) of zirconium beads was added, tubes were sealed and placed in a Bullet Blender™24 (Next Advance Inc., NY, USA) for two 5-minute cycles at speed setting of "nine" (the blender offers 10 speed levels) for adequate homogenisation of the muscle tissue. The Bullet Blender™ uses the power of a circulating ball that hits tubes with high velocity causing the rapid destructive motion of zirconium beads to finely destroy tissue samples. Tubes were checked and shaken between the two cycles.

5. Since preliminary work suggested an additional dilution of samples, a supplementary volume (i.e.1200 µL) of ice-cold homogenisation buffer was added to dilute samples to a workable level.

6. Finally, tubes were placed into a laboratory centrifuge, model Universal 320 R (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). The operational temperature of the centrifuge was always kept at 4°C. The first centrifugation was set to 10000 RPM (rotations per minute) for a duration of 5 minutes.

7. After centrifugation 100 µL of clear supernatant was taken and distributed into sets of 200 µL strip tubes labelled for CCO, CS and HOAD. Tubes were sealed, snap–frozen in liquid nitrogen and stored at -80°C.

8. In the meantime the rest of the extracts were put back for the second centrifuge set to 12000 RPM for 10 minutes. The supernatant produced was used for PK and LDH activity determination. For PK the supernatant had to be diluted 25 times which was accomplished by mixing 8 µL of the extract with 192 µL homogenisation buffer in a 200 µL tube. For LDH a 50 times extra dilution was required and thus 4 µL supernatant was diluted with the 196 µL buffer. Again, tubes were sealed, snap frozen in liquid nitrogen and taken to the -80°C freezer for storage.

All enzyme extracts were taken in duplicate in case any assay repetition was needed. This also applied for the tissue metabolites.

5.2.3.1.2 RM enzyme extraction protocol

In RM, activity of the same enzymes as in WM were scheduled for determination.

The protocol was in its essence the same as for WM with only a few differences. When RM samples were prepared for homogenisation additional care was taken to clean the sample thoroughly of any remnants of attached WM and fat tissue. Another consideration was related to relatively small quantities of RM in snapper at the first 2–3 months of the growth experiment. However, since RM mitochondrial enzyme activity is much higher than in WM, a smaller amount of RM was sufficient for the analysis. Therefore, the protocol was adjusted to work with ~50 mg RM samples for homogenisation and it was set up as a standard for all biochemical work for both species. RM enzyme extraction for determination of CCO, CS and HOAD activity was the same as for WM except the final dilutions for CCO (i.e. 15 times dilution in the homogenisation buffer), CS (i.e. only for YEM the supernatant had to be diluted an extra two times) and no extra dilution for HOAD was required. Furthermore, preliminary trials also indicated the importance of several freeze – thaw cycles, which contributed to final mitochondria membrane rupture and the consequent release of enzymes into the supernatant. Since three freeze – thaw cycles showed the highest level of enzymatic activity (there was no difference between the third and fourth cycle) this was adopted and carried out. After the second centrifugation, an extra dilution of 25 times was sufficient for both PK and LDH enzyme extracts.

5.2.3.1.3 Intestines extraction protocol

In intestines, the activity of CCO and CS were determined.

Before homogenisation, intestines were thoroughly cleaned of any attached fat remnants and also of their content. The main difference between the homogenisation buffer recipe for WM/RM and the intestine was in the requirement for 0.1 mM PHSF (phenylmethane-sulfonyl fluoride) as an inhibitor of serine protease. Another adaptation for enzymatic extraction from the intestine based on preliminary trials, was to use lower RPM during centrifugation. More specifically, 5 minutes at 1500 RPM was shown to preserve the highest enzymatic activity in the intestines extract. The supernatant was aliquoted for the enzymes of interest and no extra dilution was required. The rest was the same as explained previously.

5.2.3.1.4 Pyloric caeca extraction protocol

In the pyloric caeca, the activities of trypsin (TRY) (E.C. 3.4.21.4) and chymotrypsin (CHY) (E.C. 3.4.21.1) were determined.

Pyloric caeca (four to five finger-like formations in snapper; and two in YEM) were first cleaned of any fat residues and together with the content finely chopped and all well mixed. Preliminary investigations indicated that the best approach for protease activity determination was when both the body and the content of pyloric caeca were included in the sample. In 2 mL tubes ~100 mg pyloric caeca mash was placed together with zirconium beads and 450 µL of nano-pure water (~18 MΩ x cm). Tubes were sealed and run in the Bullet Blender™ with the same settings as for muscle preparation. Before centrifugation extra dilution was required and 450 µL nano-pure water, the muscle homogenisation buffer and Tris-HCl Trypsin reaction buffer (see 5.2.4.1.6) were added making in total 1.8 mL of solution diluting ~100 mg of pyloric caeca mixture. The centrifuge was set at 12000 RPM for 10 minutes. The supernatant was divided for the two enzymatic activity assays in the same way as described earlier and there was no need for additional dilution.

5.2.3.1.5 Liver extraction protocol

In the liver only HOAD activity was determined

The homogenisation was the same as for muscles with the difference that only one cycle in the Bullet Blender™ was required and the centrifuge was set up at 10,000 RPM for five minutes. No extra dilution was required for the HOAD assay.

5.2.3.2 Sample preparation for tissue metabolites measurement

The tissues of interest for measurement of stored metabolites were WM, RM, and liver. The metabolites of interest were tissue lactate, glucose and glycogen. Protocols described in this section were based on Tuckey et al. (2012), with minor modifications due to differences between the two studies in terms of different equipment and species used.

5.2.3.2.1 Sample homogenisation and perchloric acid (PCA) tissue extractions

Reagents used in this method were:

- 0.4 M perchloric acid ($HClO_4$)
- 3 M Potassium hydroxide (KOH)/1 M Potassium carbonate (K_2CO_3)

Stages of metabolite extraction:

1. Tissue samples were brought from the -80°C freezer and kept in the -20°C lab freezer for the duration (~1 hour) of the sample preparation.

2. Tissue samples were prepared in the same way as described earlier, minced with the scalpel blade and ~100 mg was placed into a 2 mL eppendorf tube and its mass was recorded.

3. Into each 2 mL tube 400 µL of ice-cold 0.4 M PCA and a scoop of zirconium beads were added.

4. The tubes were placed in the Bullet Blender™ for two cycles of 5 minutes at speed setting of "nine".

5. After tissue destruction the tubes were placed on the cooling block and 100 µL of crude homogenate was taken and placed in 1.5 mL eppendorf tubes for the digestion step required for glycogen analysis (see 5.2.4.2).

6. The rest of the crude homogenate was centrifuged at 10000 RPM for 5 minutes.

7. After centrifugation 150 µL of supernatant was removed and placed in a 1.5 mL tube. For acid neutralisation (to approximately pH 6.5) 11.3 µL 3 M KOH/1 M K_2CO_3 were added into tubes and vortexed on a small orbital shaker, model MS1 Minishaker (IKA Works GmbH & Co. KG in Staufen, Germany) for 20 seconds and placed on the cooling block for 5 minutes.

8. Tubes were then put into the centrifuge at 10000 RPM for 5 minutes.

9. Supernatant was aliquoted into separate 200 µL strip tubes labelled for lactate and glucose determination.

Dilution specifics per tissue type and species:

- Snapper lactate: WM required an extra 12 times dilution in nano-pure water; RM required an extra 10 times dilution; for liver there was no need for an extra dilution.

- Snapper glucose: for white and RM no extra dilution was required; liver samples needed to be diluted an additional 10 times.

- YEM lactate: WM required an extra 20 times dilution; RM 10 times and for liver an extra two times dilution was required.

- YEM glucose: white and RM required an extra two times dilution; and liver required an extra 10 times dilution.

Once all samples were obtained they were snap frozen in liquid nitrogen and stored at -80°C for subsequent analysis.

5.2.3.2.2 Tissue digestion for glycogen determination

As explained in 5.2.3.2.1, 100 µL of crude (i.e. not centrifuged) homogenate was taken from each sample tube for tissue digestion mandatory for the glycogen determination. The crude homogenates did not undergo centrifugation because the impact of the centrifugal force would cause all remaining fragments of tissue sample, including glycogen to be eventually discarded. Homogenisation in the Bullet Blender™, as any other mechanical homogenisation procedures are not capable of breaking the glycosidic bonds that lock and therefore store glucose molecules in the multibranched polysaccharide glycogen (Fig. 5.1). However, these bonds are readily broken down by the catalytic activity of the enzyme amyloglucosidase at 37°C (Keppler and Decker, 1974). Therefore, after the digestion of the crude tissue samples with amyloglucosidase the extract contains glucose molecules from two sources, one from the pool of "free" tissue molecules liberated by the Bullet Blender and the other originating from glucose stored in glycogen. These combined represent the total glucose in the sample that can now be measured by the glucose determination procedure.

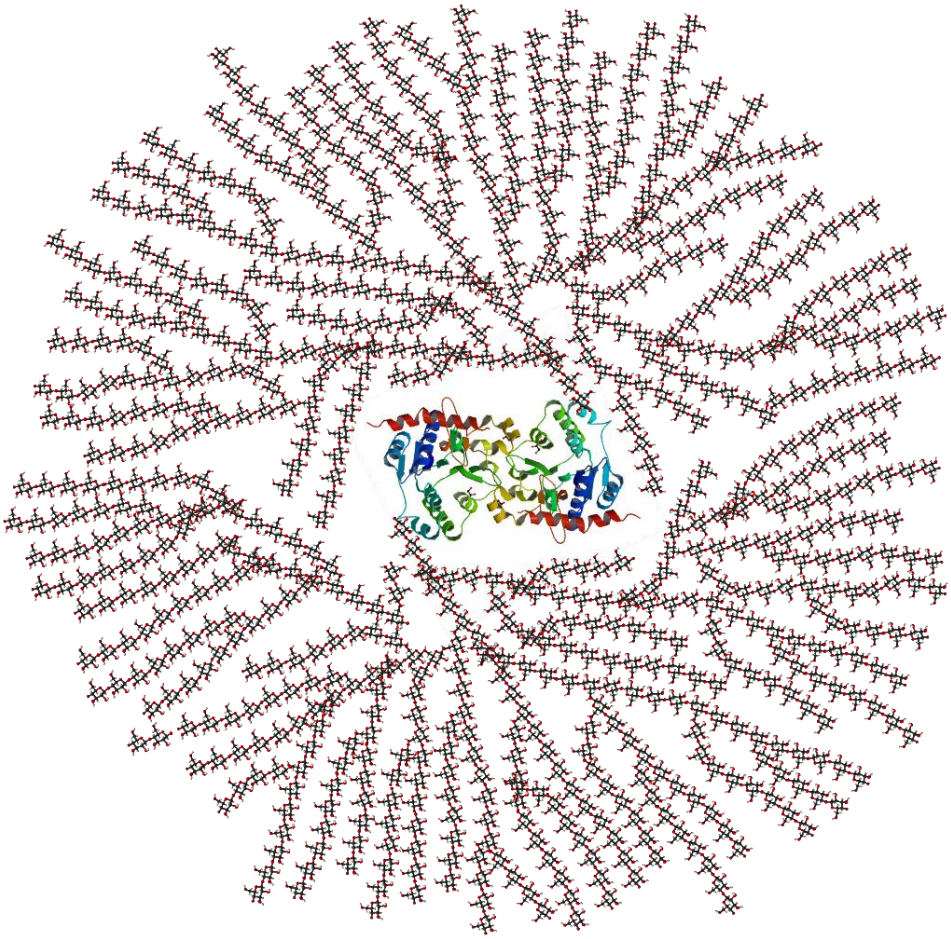

Figure 5.1. Schematic illustration of glucose molecules branched from the protein core, bonded into multibranched formation of glycogen. Picture obtained from Wikipedia – The Free Encyclopaedia (https://en.wikipedia.org/wiki/Glycogen).

Reagents used for digestion:

- 0.2 M sodium acetate ($C_2H_3NaO_2$), pH 4.8
- 2 mg mL^{-1} amyloglucosidase (from *Aspergillus niger*)

Digestion protocol:

1. In 1.5 mL eppendorf tubes with 100 µL of crude PCA extract 10 µL of 3 M KOH/1 M K2CO3 was added and vortexed for 10 seconds to neutralise the acid component to approximately pH 6.5.

2. 500 µL of 2 mg/mL amyloglucosidase solution prepared in 0.2 M sodium acetate was added to the tubes and vortexed for 10 seconds.

3. Once all samples per batch went through step 1 and 2, they were placed on the thermo-mixer, model Comfort by Eppendorf (Eppendorf AG, Hamburg, Germany) set at 37°C for 2.5 hours with the shaking option employed at 350 RPM.

4. After digestion tubes were placed on the cooling block for 5 minutes and centrifuged at 10000 RPM for 5 minutes.

5. Clear supernatant was removed and further diluted with nano-pure water as follows:

- Snapper glycogen: WM did not need any extra dilution; RM required two and liver required 12 times extra dilution.

- YEM glycogen: WM did not need any extra dilution; RM required two and liver 10 times extra dilution.

6. Samples were snap-frozen and stored at -80 °C until required for analysis.

5.2.4 Determination of enzyme activities and quantification of tissue metabolites in homogenate extracts

5.2.4.1 Enzyme activity determination

Enzyme activities and metabolite levels in selected tissues were measured spectrophotometrically in the multifunctional microplate reader, model CLARIOstar® (BMG LABTECH GmbH, Ortenberg, Germany). The operational temperature was set to 25°C for all tests, unless otherwise stated. For all measurements the corresponding CLARIOstar® software (v 5.01) was programmed to run in the absorbance plate mode. The discrete wave length setting was determined and set to suit spectral requirements for kinetics measurements of particular enzymes. Reaction buffer solutions were placed in the Y14 Circulating water bath (Grant Instruments, Cambridge, UK) set at 25°C to acclimate for the final preparation of plates before the colorimetric measurement of enzyme kinetics. The plate type used for all assays was clear GREINER 96 flat bottom microplates. The general principle was to measure kinetics of enzymes in the first few minutes of reaction time when the initial portion of the generated slope was the steepest and still linear (i.e. maximum linear rate) either in the positive or negative direction portrayed as the classic Michaelis–Menten kinetic curve. Nevertheless, non-Michaelis–Menten kinetics were observed when activities of CS were measured. The CLARIOstar® MARS Data Analysis software (v 3.00 R3) was utilised to display kinetics in OD (optical density) values per selected unit of time (i.e. minute^{-1}), and for any subsequent data manipulation. The volume of

reaction buffer per microplate well for all assays was standardised at 200 µL for uniform operation. Assays were done either in duplicate if preliminary results showed difference in repeatability of samples < 10%, or in triplicate when the differences in measurements of the repeated samples were more than 10% on average.

The final calculation of enzymatic activity was performed with the following equation:

$$U\ g^{-1} = \frac{\Delta A\ min^{-1} *\ Di * Vt * Df}{\varepsilon * Vt}$$

$U\ g^{-1}$ – the enzyme activity unit expressed in µmol min^{-1} g wet $tissue^{-1}$

$\Delta A\ min^{-1}$ – optical density absorbance measurement corrected for the blank/control value $minute^{-1}$

Di – the initial dilution factor calculated as:

$$Di = \frac{sample\ mass\ (g) + buffer\ volume(mL)}{sample\ mass\ (g)}$$

Vt – total assay volume $well^{-1}$ (mL)

Df – the final dilution, if any, as was specified in 523 under the different tissue extraction protocol

ε – extinction (attenuation) coefficient is a measurement of how strongly a substance attenuates light at a given wavelength, expressed in $mM^{-1}cm^{-1}$ unless stated differently

Vs – volume of sample within the total assay volume (mL)

Extinction coefficient is typically expressed per 1 cm length. In the case of spectrophotometry where 96 microplates are used the length that light travels through the assay solution is less than 1 cm (e.g. 0.2 cm); however, the capability of the CLARIOstar® plate reader was to automatically convert whatever the length was specified in the set-up panel for the colorimetric measurements to recalculate the OD values to the standardise length of 1 cm.

Cytochrome *c* oxidase

CCO activity was determined in three tissues (white and RM, and intestine) of both species by the use of a commercial CCO assay kit (Sigma-Aldrich, St. Louis, MO, USA). The original protocol supplied with the kit was modified to suit a design that was based on preliminary trials, which differed slightly amongst tissue types and species.

Conditions of the CCO assay:

- 10 mM Tris-HCl, pH 7.0, containing120 mM KCl
- 10 mM Tris-HCl, pH 7.0, containing 250 mM sucrose
- 0.22 mM Ferrocytochrome *c* Substrate Solution
- 0.1 M Dithiothreitol (DTT) Solution

The principle of the assay: The cytochrome *c* protein as purchased was in its oxidised (ferric) form and the first step was to reduce it with DTT into the ferrous state. The reduction was observed by change in the colour of the solution from dark red to pale purple and its level was determined by measuring the absorbance ratio between two wavelengths 550 and 560 nm in the microplate reader set to the absorbance mode. The workable ratio was between 10 and 20. The principle of the assay is based on the colorimetric observation of the decrease in absorbance at 550 nm when ferrocytochrome *c* is oxidised to its ferric state through the catalytic activity of CCO. Thus, the activity unit of CCO may read as the amount of enzyme required to oxidise 1.0 µmol of ferrocytochrome *c* per minute at 25°C and pH 7.0. To calculate kinetics of the enzyme the millimolar extinction coefficient (ε) between reduced and oxidized cytochrome *c* protein of 21.84 $mM^{-1}cm^{-1}$ at 550 nm was used. During the assay the initial rapid fraction (steep and linear part of the slope) of the reaction within approximately the first 40 seconds was considered to represent the activity of the enzyme. To establish validity of the protocol the blank and positive control assay were regularly carried out.

Citrate synthase

CS was determined in the same tissue types as CCO. The assay was based on catalytic performance of CS described as:

$$\text{Acetyl CoA + Oxaloacetate} \xrightarrow{\text{CS}} \text{Citrate + CoA-SH + } H^+ + H_2O$$

And the colorimetric reaction:

$$\text{CoA-SH + DTNB} \longrightarrow \text{TNB + CoA-S-S-TNB}$$

The products of the reaction between Acetyl CoA with Oxaloacetate catalysed by CS are the six-carbon product citric acid, coenzyme A with a thiol (SH) group, a water molecule and a proton. The CS

activity unit therefore can be described as the quantity of enzyme required to catalyse the hydrolysis of 1.0 μmol of acetyl CoA per minute at pH 7.1 in the presence of oxaloacetate at 25°C. Once CoA-SH was produced by the hydrolysis of the thioester of acetyl CoA, it reacts with 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB) and forms 5-thio-2-nitrobenzoic acid (TNB). Presence of TNB in the assay is stoichiometrically proportional to the amount of produced citrate and CoA-SH and it is spectrophotometrically detected by measuring absorbance at 412 nm.

Conditions of the CS assay:

- 20 mM TRIS, pH 7.1
- 0.1 mM 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB) in 95% ethanol
- 0.3 mM Acetyl-CoA ($C_{23}H_{35}N_7O_{17}P_3SLi_3$)
- 0.5 mM Oxaloacetate in 20 mM TRIS buffer (omitted for the control)

In a sample there are often some other products, predominantly thiolase and deacetylase, that also react with Acetyl CoA and generate CoA-SH which in turn react with DTNB contributing to production of TNB. Therefore, it was important to account for the event by regularly running control assays to be able to establish the level of other CoA-SH producing elements and correct the final activity kinetics accordingly. The CS assay kinetics for all tissue types exhibited non-Michaelis–Menten kinetics. The kinetics is distinguished for a sigmoid curve indicating cooperative binding of substrate to the active site, as binding of one substrate molecule triggers the binding of subsequent substrate molecules. Therefore, CS assays were running for up to 15 minutes and the steepest linear portion of the response curve (on average between 3rd and 10th minute) was evaluated and utilised for the activity determination. The extinction coefficient of TNB at 412 nm used was 13.6 mM^{-1} cm^{-1}.

3-hydroxyacyl-CoA dehydrogenase

HOAD activity determination was measured in white and RM, and liver. Principle of the assay is based on the following reaction:

$$\text{S-Acetoacetyl-CoA + ß-NADH} \xrightarrow{\text{HOAD}} \text{3-Hydroxybutyryl-CoA + ß-NAD+,}$$

where ß-Nicotinamide Adenine Dinucleotide (ß-NADH) is converted into its oxidised form ß-NAD+. Therefore, the activity unit can be defined as conversion of 1.0 μmol of acetoacetyl-CoA to ß-hydroxybutyryl-CoA that occurs per minute at pH 7.3 in the presence of ß-NADH. This reaction is observed spectrophotometrically by following the oxidation of ß-NADH at 340 nm. Millimolar extinction coefficient of ß-NAD at 340 nm was 6.22 mM^{-1} cm^{-1}.

Conditions of the HOAD assay:

- 50 mM imidazole, pH 7.3
- 0.15 mM NADH
- 0.1 mM Acetoacetyl-CoA (omitted for the control)

Pyruvate kinase

Determination of PK activity was required in white and RM. The principle of the assay was centred on the following two reactions:

$$\text{Phosphoenolpyruvate (PEP)} + \text{ADP} \xrightarrow{\text{PK}} \text{Pyruvate} + \text{ATP}$$

L-Lactate Dehydrogenase (LDH):

$$\text{Pyruvate} + \beta\text{-NADH} \xrightarrow{\text{LDH}} \text{L-Lactate} + \beta\text{-NAD+}$$

Pyruvate that was created from PEP by catalytic performance of PK was measured by the formation of NAD+ in the presence of LDH, which is in sequence stoichiometrically proportional to the amount of the created pyruvate. Therefore, the unit of activity can be described as the amount of enzyme required to transfer a phosphate group from PEP to ADP to produce 1.0 µmol of pyruvate in one minute at 25 °C and pH 7.4.

Conditions of the PK assay:

- Imidazole Stock (50 mM imidazole, 100 mM KCl and 20 mM $MgCl_2$), pH 7.4
- 5 mM ADP, 0.15 mM NADH and 10 IU of LDH (550 U/mg as 10 g suspension in 2 ml of 3.2 M ammonium sulfate at 25°C) made fresh daily in the Imidazole Stock for the control
- 5 mM PEP added to the above cocktail for the Reaction Buffer

The extinction coefficient for β-NAD+ was the same as for HOAD activity determination (6.22 mM^{-1} cm^{-1}).

Lactate dehydrogenase

LDH activity was determined in white and RM. The assay protocol with its components was a modified version of the LDH Assay by Worthington Enzyme Manual (Worthington and Worthington, 2011). The principle of the assay is the same as depicted in the second reaction for PK activity determination:

$$\text{Pyruvate} + \beta\text{-NADH} + H^+ \xrightarrow{\text{LDH}} \text{L-Lactate} + \beta\text{-NAD+}$$

So, the unit of LDH activity can be defined as the amount of enzyme needed to reduce 1 µmol of pyruvate to L-lactate per minute at 25°C.

Conditions of the LDH assay:

- 0.2 M Tris-HCl, pH 7.3
- 0.22 mM NADH in 0.2 M Tris-HCl buffer
- 1.17 mM Sodium pyruvate in 0.2 Tris-HCl buffer

The millimolar extinction coefficient, again, the same as for PK and HOAD determination of 6.22 $mM^{-1}cm^{-1}$ was utilised for the activity calculation.

Trypsin

TRY and CHY activity was determined in the pyloric caeca of the test species based on a protocol adapted from Yetty et al. (2004) as originally developed by Erlanger et al. (1961). The substrate for the TRY assay was benzoyl-DL-arginine-p-nitroanilide (BAPNA).

Conditions of the TRY assay:

- Tris-HCl stock made of 50 mM Tris-HCl and 20 mM $CaCl_2$ x $2H_2O$, pH 8.1
- 1 mM BAPNA
- 1% (v/v) DMSO (dimethyl sulfoxide)

The stock BAPNA solution was made in DMSO by dissolving 43.5 mg BAPNA in 1 mL DMSO. On the day the required volume of reaction buffer was produced by diluting the stock BAPNA 100–fold with previously made Tris-HCl stock buffer. The absorbance of the catalytic reaction that released p-nitroaniline from BAPNA was then determined at 410 nm and one enzyme unit was expressed as the amount of enzyme utilised to hydrolyse 1 µmol of BAPNA under the assay conditions $minute^{-1}$. The extinction coefficient of p-nitroaniline released from chromogens of BAPNA used for final calculation of TRY activity was 8800 $M^{-1}cm^{-1}$. The difference in the extinction coefficient unit required a correction factor of 1000 in the numerator of the 5241 equation to bring the final unit to the standard of the entire chapter (i.e. 1 µmol of substrate converted $minute^{-1}$).

Chymotrypsin

The substrate for the assay was succinyl- (Ala)2-Pro-phe-p-nitroanilide (SAPNA).

Conditions of the CHY assay:

- Tris-HCl stock made of 50 mM Tris-HCl and 20 mM $CaCl_2$ x $2H_2O$, pH 8.4
- 0.1 mM SAPNA

The reaction buffer was made fresh with mixing and heating due to SAPNA's slow dissolving properties. All other characteristics of the assay were the same as for TRY.

5.2.4.2 Determination of stored tissue metabolites

The state of tissue metabolites of interest was determined using commercial L-lactate (K-late 07/14) and D-glucose-HK (K-gluhk-220A 07/14) kits from Megazyme (Megazyme International Ireland, Wicklow, Ireland). The protocols supplied with the kits were used as the general guideline, including the steps required for the assays and any specifics of the tissues and/or species were adopted to the protocol based on the preliminary investigation. Per batch of samples all assays were carried out by constructing a full calibration or standard curve performed concurrently using the same batch of reagents. The microplate reader was set to the absorbance *end point* mode and was loaded with the standard concentrations values in μmol L^{-1}.

Lactate

The principle of L-lactate determination:

There are two steps in quantifying L-lactate in samples. The first is the same as used for determination of LDH activity rates but with the reaction in the opposite direction:

$$\text{L-Lactate} + \beta\text{-AND} + \underset{\longleftarrow}{\overset{\text{LDH}}{\longrightarrow}} \text{Pyruvate} + \beta\text{-NADH} + H^+$$

L-lactate is oxidised in the presence of NAD to pyruvate. However, the equilibrium moves towards the left side of the reaction which contributes to the short lifespan of the pyruvate. Therefore, pyruvate has to be captured in order to keep the right part of the reaction stable. This is accomplished by adding two components to the reaction mixture: D-glutamate and enzyme D-glutamate-pyruvate transaminase (D-GPT):

$$\text{Pyruvate} + \text{D-glutamate} \xrightarrow{\text{D-GPT}} \text{D-alanine} + \text{2-oxoglutarate},$$

This locks pyruvate permanently and NADH that is stoichiometric with the original amount of L-lactate is then measured as an increase in absorbance at 340 nm.

Preliminary exploration assisted in determining dilution factors for different tissues and species in such a way that any expected results would fit within the standard curve of the assay. The final unit in which data were presented was in μmol g^{-1} wet tissue, and the equation utilised for the quantification of L-lactate was:

$$TC = \frac{SC * Df}{STC * 1000}$$

TC – Tissue Concentration in μmol g^{-1} of wet tissue mass

SC – Sample Concentration in μmol L^{-1}derived form the linear regression equation representing the standard curve fitting.

Df – Dilution factor that was implemented in order to expect the sample concentration measurements to fit within the standard curve

1000 – the correction factor to bring the final unit to μmol g^{-1}

STC – final sample tissue concentration (g mL^{-1}) after neutralisation of PCA extract calculated as:

$$STC = STCbn\frac{0.150}{0.150 + 0.0113}$$

0.150 – the volume (mL) of supernatant recovered after the centrifuge

0.0113 – the volume (mL) of the neutralisation agent (i.e. 3 M KOH/1 M K_2CO_3)

$STCbn$ – sample tissue concentration (g mL^{-1}) before neutralisation calculated as:

$$STCbn = \frac{SM}{SM + 0.4}$$

SM – mass of samples in grams

0.4 – the volume (mL) of cold PCA in mL added at the beginning of the metabolites extraction

Glucose

Principle of D-glucose determination:

There are two distinct steps to evaluating the amount of D-glucose in the given tissue sample via the Megazyme kit. First, enzyme hexokinase (HK) utilises a molecule of ATP (adenosine-5-triphosphate) to convert D-glucose to glucose-6-phosphate (G6P):

$$\text{D-glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{G6P} + \text{ADP}$$

Another enzyme, glucose-6-phosphate dehydrogenase (G6P-DH) catalyses the oxidation of G6P with NADP+ (nicotinamide-adenine dinucleotide phosphate) forming gluconate-6-phosphate (GN6P) and NADPH:

$$\text{G6P} + \text{NADP+} \xrightarrow{\text{G6PDH}} \text{GN6P} + \text{NADPH} + \text{H}^+$$

In the same manner as for L-lactate, since the amount of reduced NADPH is stoichiometrically proportional to the initial amount of D-glucose, so by measuring the increase in absorbance at 340 nm, D-glucose present in the given sample is determined. The equation for determination of tissue concentration of D-glucose was the same as for L-lactate including the units that glucose was expressed (i.e. μmol g^{-1} of wet tissue mass).

Glycogen

Stored tissue glycogen was examined coupled with determination of tissue glucose as was shown in 5.2.4.2. In essence, the spectrophotometric component of glycogen determination is the same as for glucose. The only difference was in the final calculation step where the total glucose value that originated from the digested samples, which included "free" glucose plus digested glycogen glucose, was subtracted from glucose found in the same but not digested sample. Therefore, glycogen stored in a given tissue was expressed in μmol glucosyl units g^{-1} of wet tissue mass.

5.2.5 Enzymatic activity and tissue metabolites data processing and calculation

5.2.5.1 Enzymatic activity and tissue metabolites

Data for activities of selected enzymes and tissue metabolites were gathered from samples obtained during the monthly/bimonthly measurements sessions throughout 12-month feeding trials. Thus, the general processing and calculations were identical to data for supplementary morphometrics (i.e. organosomatic and related indices, chapter 2).

5.2.5.2 Enzymatic ratios

To express enzymes activities in the form of ratios is an advantageous approach as it allows comparisons between tissues and species, particularly when absolute values are widely different (Hochachka et al., 1982). Therefore, to examine differences between snapper and YEM and to identify preferable metabolic pathways in different tissues as affected by annual cycling when kept under unrestricted diet, a set of relevant ratios were calculated and employed for comparison. The ratios of interest were CS/COO (importance of the Krebs cycle relative to electron transport chain, ETC); HOAD/CS and HOAD/COO (relative importance of β-oxidation to aerobic metabolism and ETC respectively); LDH/CS (measure of relative anaerobic vs. aerobic capacity); and PK/LDH (importance of aerobic over anaerobic glycolysis). For the enzymatic ratios, annual mean values were calculated to identify differences in general (annual) preferences for certain metabolic pathways between two species.

5.2.6 Statistical methods

General statistical approaches used for enzyme activities, concentrations of selected tissue metabolites and enzymatic ratios data obtained for the 12-month experimental period, were the same as described in chapter 2 (section 2.2.6.5) for supplementary morphometrics.

In addition, for ratios only, annual mean values were compared between tissues and species with two-way ANOVA in a manner described in 2.2.6.1.

5.3 Results

5.3.1 Snapper and YEM enzymatic activity – effects of temperature

5.3.1.1 Cytochrome *c* oxidase (CCO) – correlation with temperature and between tissues

5.3.1.1.1 Snapper

Snapper CCO activity in the selected tissues exhibited frequent changes through the 12-month period, which was not generally associated with annual sea-water temperature oscillations (Fig. 5.2A, B, C). Nevertheless, significant correlation with temperature was found in white muscle (WM, Table 5.1 Fig. 5.2A). However, since the lag time phenomenon was present, CCO only correlated when activity data were moved a data point forward along the temperature curve showing a moderately strong, positive correlation (Table 5.1). In contrast, although a negative correlation with temperature could be assumed based on a graphical illustration (Fig. 5.2B, C), CCO activity in red muscle and intestinal tissue was not significantly associated with temperature (Table 5.1). However, similar trajectories of CCO activity between red muscle (RM) and intestines pointed to a positive correlation between the two tissues which was statistically confirmed for both raw and predicted data (raw, $R = 0.844$, $R^2 = 0.714$, $F_{1, 9} = 19.95$, $p = 0.002$; predicted, $R = 0.940$, $R^2 = 0.883$, $F_{1, 9} = 60.20$, $p < 0.001$).

5.3.1.1.2 YEM

Trajectory of annual YEM CCO activity differed amongst the three selected tissues (Fig 5.2D, E, F). Generally, there was no correlation between CCO activity and annual temperature oscillation, apart from the activity measured in intestines, where positive association was observed (Table 5.2, Fig. 5.2F). In terms of inter-tissue correlation only CCO activity in white muscle was significantly positively correlated with the activity in YEM intestines (raw – $R = 0.725$, $R^2 = 0.526$, $F_{1, 12} = 12.21$, $p = 0.005$, predicted – $R = 0.756$, $R^2 = 0.572$, $F_{1, 12} = 14.72$, $p = 0.003$).

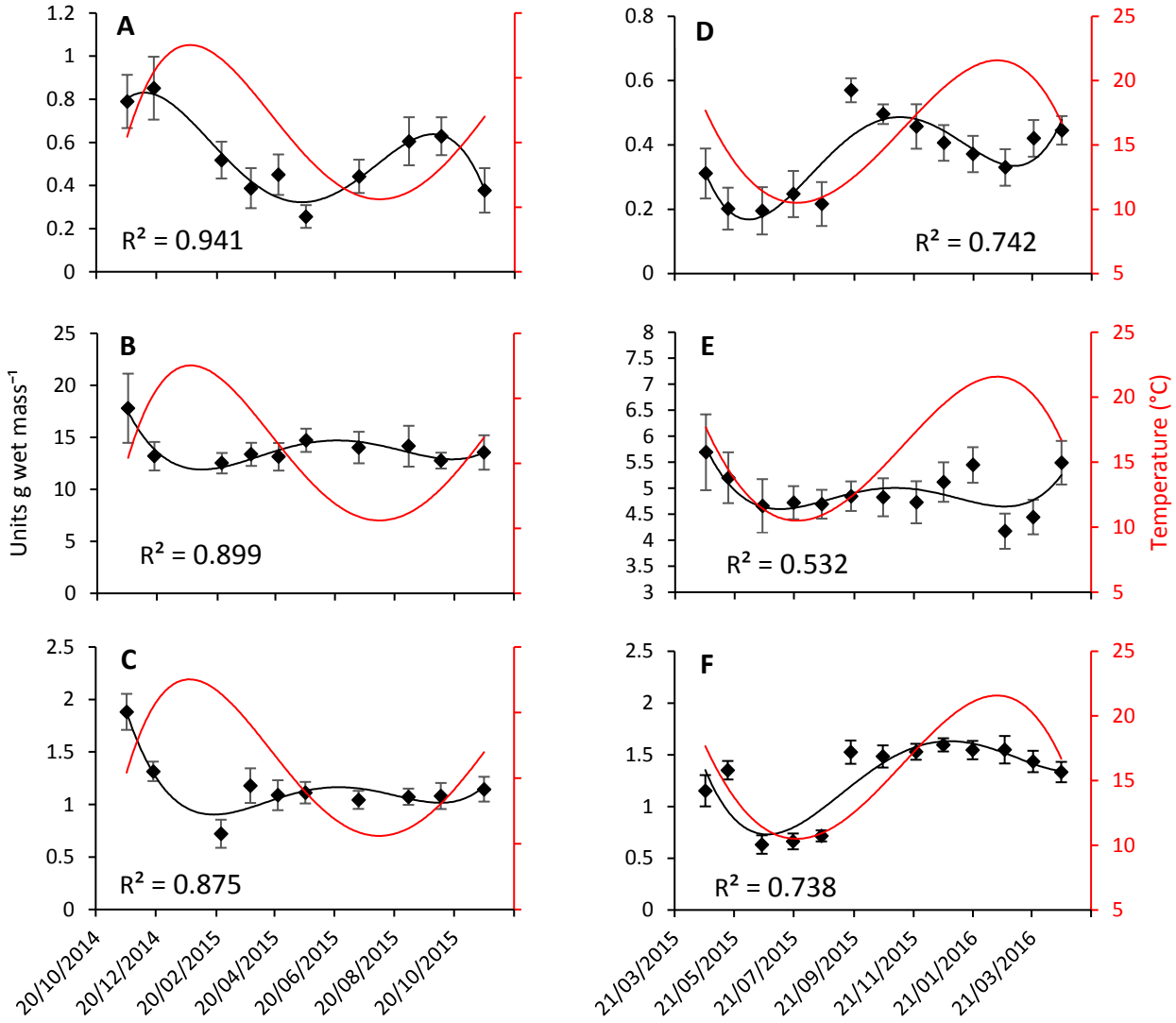

Figure 5.2. Cytochrome *c* oxidase (CCO) activity (black curve and black symbols) for 12-month experimental period superimposed with sea-water temperature curve (red), in snapper tissue (A – white muscle, B – red muscle, C – intestines) and YEM tissue (D – white muscle, E – red muscle, F – intestines). Curves were derived from raw data fitted to 4th polynomial function (temperature data points omitted for clarity). R^2 belongs to the 4th polynomial function fitted to the data. Error bars are 95% confidence intervals. The caption of other graphs in the chapter are equivalent to Fig. 5.2, unless stated differently.

5.3.1.2 Citrate synthase (CS) – correlation with temperature and between tissues

5.3.1.2.1 Snapper

CS activity positively correlated with temperature in WM and intestines, but there was no significant correlation observed with CS activity in RM (Table 5.1; Fig. 5.3A, B, C). Concurrently, CS activity between WM and intestines exhibited a strong positive correlation (raw – $R = 0.725$, $R^2 = 0.526$, $F_{1, 9} = 30.25$, $p < 0.001$, predicted – $R = 0.943$, $R^2 = 0.900$, $F_{1, 9} = 64.53$, $p < 0.001$).

5.3.1.2.2 YEM

YEM CS activity was not associated with sea-water temperature in any of the three selected tissues (Table 5.2, Fig. 5.3D, E, F). Unlike snapper, an inter-tissue correlation was observed between the two muscles, which was significant for both raw and predicted data (raw – $R = 0.587$, $R^2 = 0.345$, $F_{1, 12} = 5.80$, $p = 0.035$, predicted – $R = 0.930$, $R^2 = 0.862$, $F_{1, 12} = 68.85$, $p < 0.001$).

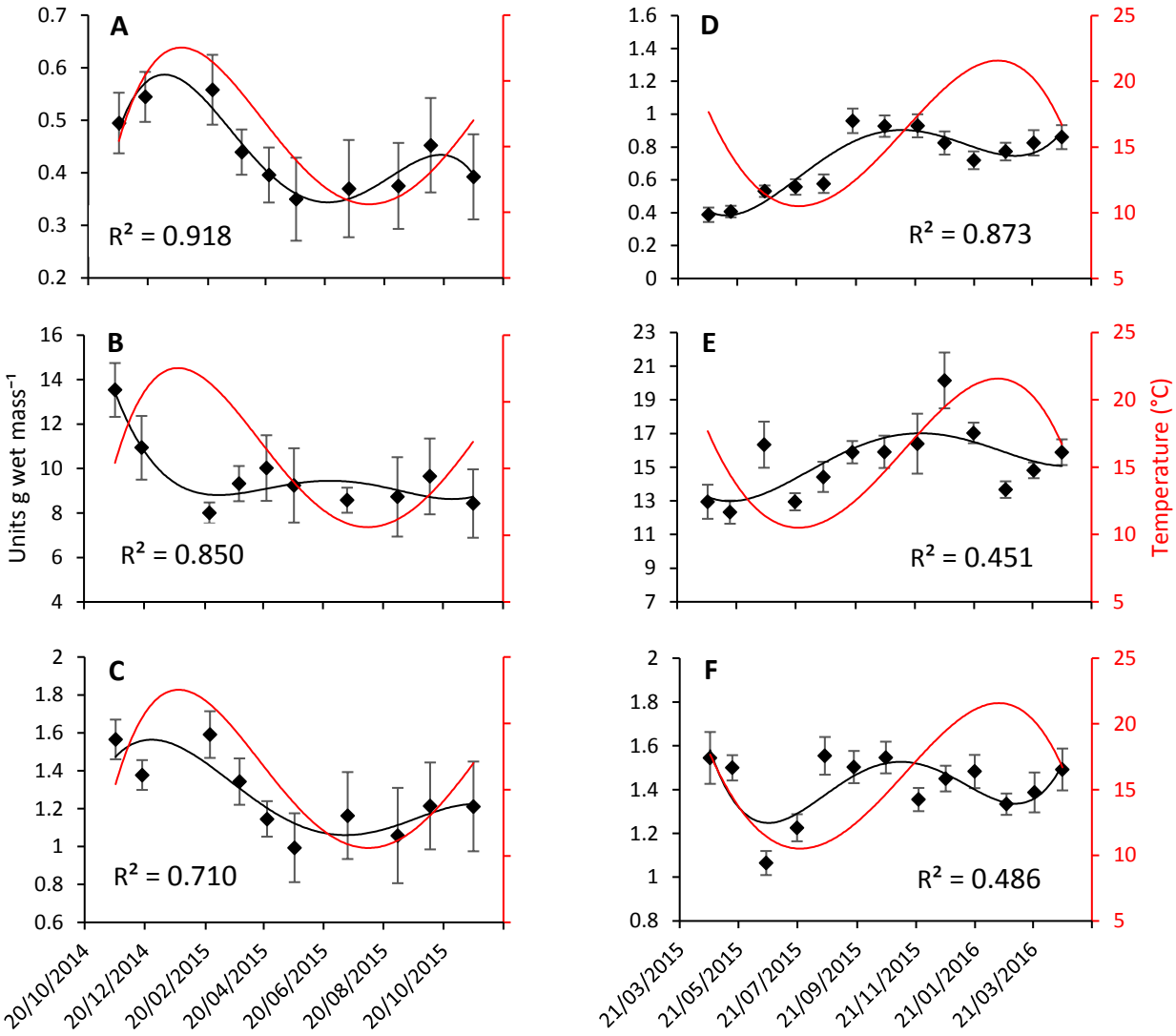

Figure 5.3. Citrate synthase (CS) activity in snapper (A – white muscle, B – red muscle, C – intestines) and YEM tissues (D – white muscle, E – red muscle, F – intestines).

5.3.1.3 *3*-hydroxyacyl-CoA dehydrogenase (HOAD) – correlation with temperature and between tissues

5.3.1.3.1 Snapper

Snapper HOAD activity was not related either to sea-water temperature or amongst tissue types (Table 1, Fig. 5.4A, B, C). In addition, liver HOAD was the only enzyme that did not show statistical differences after two-way ANOVA was performed on monthly activity data, or in other words activity of liver HOAD had a propensity to remain balanced all year around (Fig. 5.4C).

5.3.1.3.2 YEM

HOAD activity only in YEM WM was correlated (negatively) with sea-water temperature (Table 5.2, Fig. 5.4D), whereas in RM and liver this was not the case either for raw or predicted data (Table 5.2). Besides, activities did not correlate amongst the selected tissues even though, particularly in two muscle tissues, activities appeared to follow similar trajectories (predicted – R = 0.483, R^2 = 0.233, $F_{1, 12}$ = 3.36, p = 0.094).

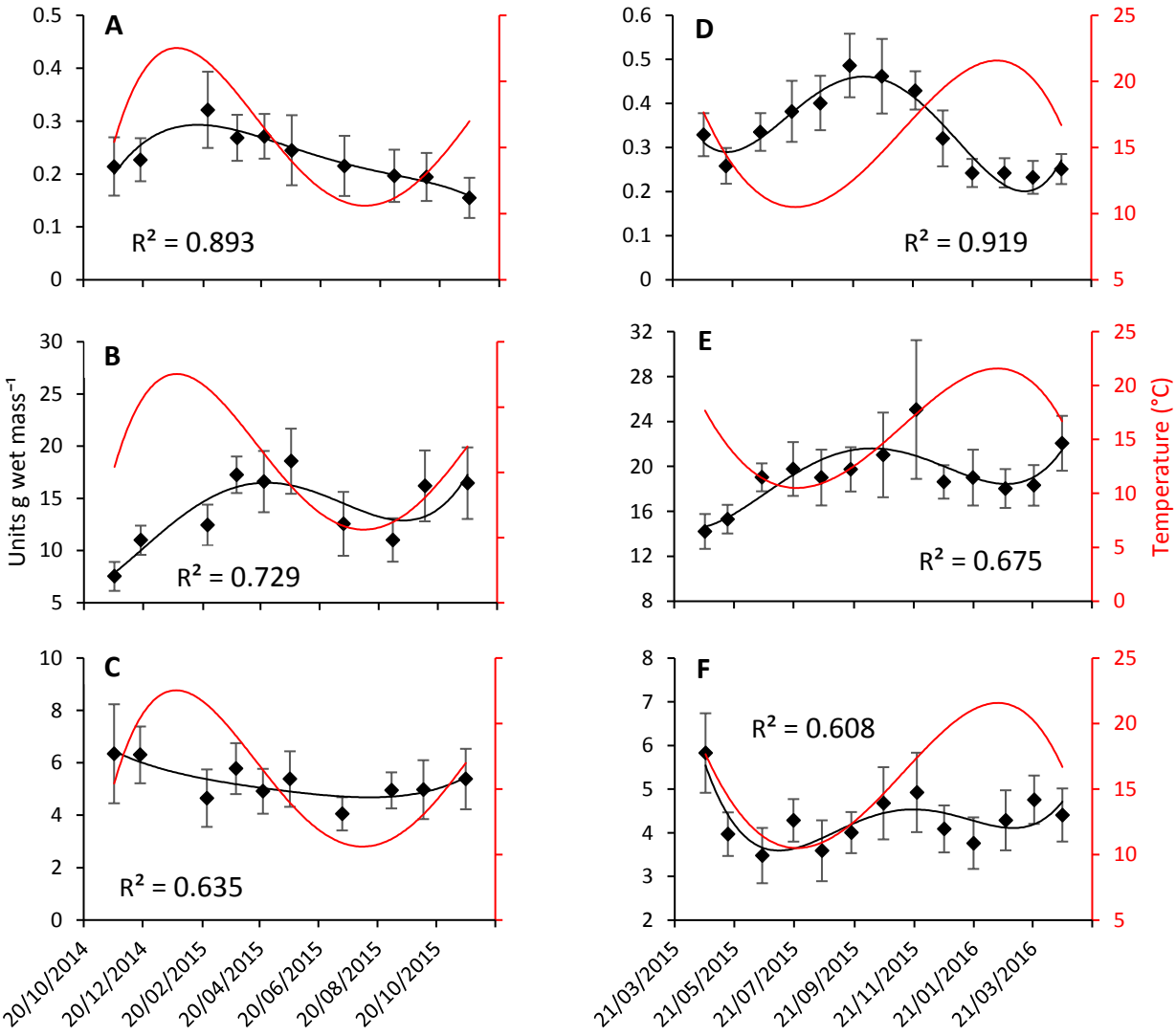

Figure 5.4. 3-hydroxyacyl-CoA dehydrogenase (HOAD) activity in snapper (A – white muscle, B – red muscle, C – liver) and YEM tissues (D – white muscle, E – red muscle, F – liver).

5.3.1.4 Lactate dehydrogenase (LDH) – correlation with temperature and between tissues

5.3.1.4.1 Snapper

LDH activity in both snapper muscle tissues did not show any particular pattern in regards to annual temperature variation (Table 5.1, Fig. 5.5A, B). Neither did LDH activities correlate between the two tissue types.

5.3.1.4.2 YEM

LDH YEM activity in WM did not correlate with annual temperature data; however, it did, negatively in RM (Table 5.2, Fig. 5.5C, D). Conversely, there was no significant correlation found between the two muscle tissues.

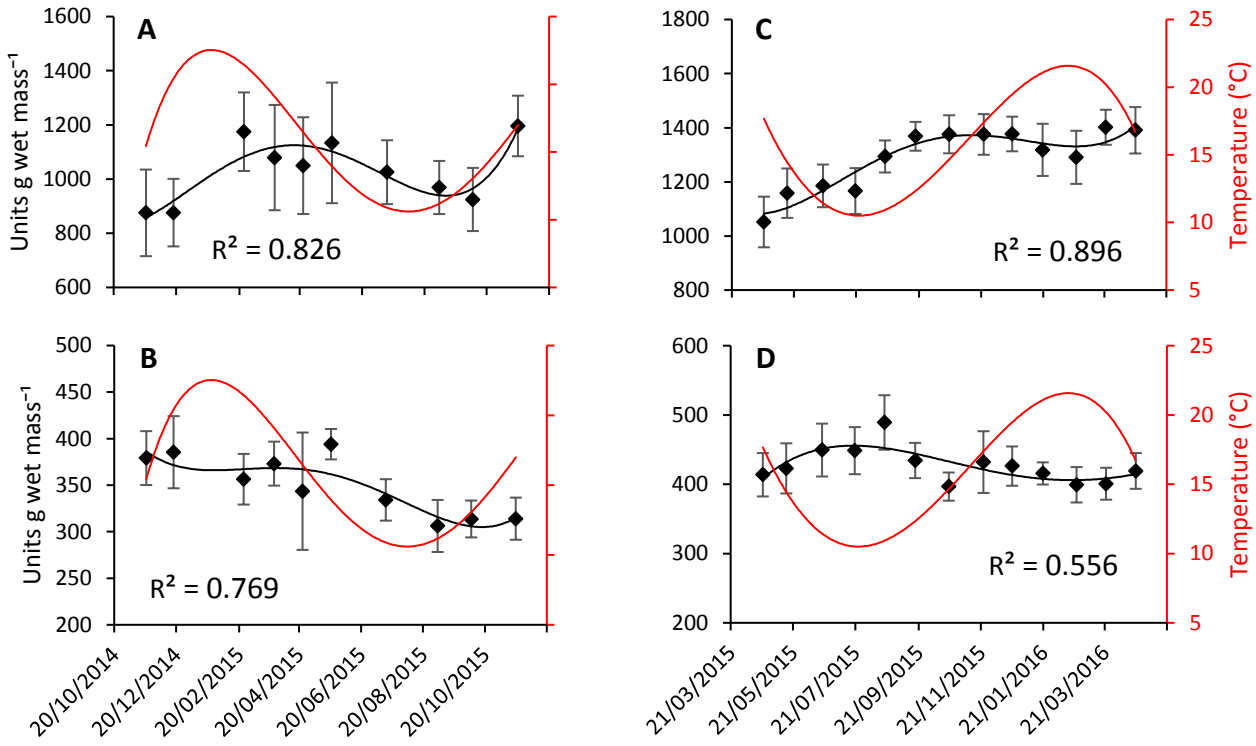

Figure 5.5. Lactate dehydrogenase (LDH) activity in snapper (A – white muscle, B – red muscle) and YEM tissues (C – white muscle, D – red muscle).

5.3.1.5 Pyruvate kinase (PK) – correlation with temperature and between tissues

5.3.1.5.1 Snapper

Activity of PK in WM was not significantly associated with sea-water temperature (Table 5.1, Fig 5.6A), whereas PK in RM was, exhibiting a moderate to strong positive correlation (Table 5.1, Fig. 5.6B). Overall, the general tendency of white muscle PK activity was to increase through the 12-month experimental period, whereas a somewhat opposite trend was observed in RM PK activity. Accordingly, a negative correlation between the two muscle tissues was found (raw – $R = 0.640$, $R^2 = 0.410$, $F_{1, 9} = 5.55$, $p = 0.046$, predicted – $R = 0.749$, $R^2 = 0.561$, $F_{1, 9} = 10.22$, $p = 0.012$).

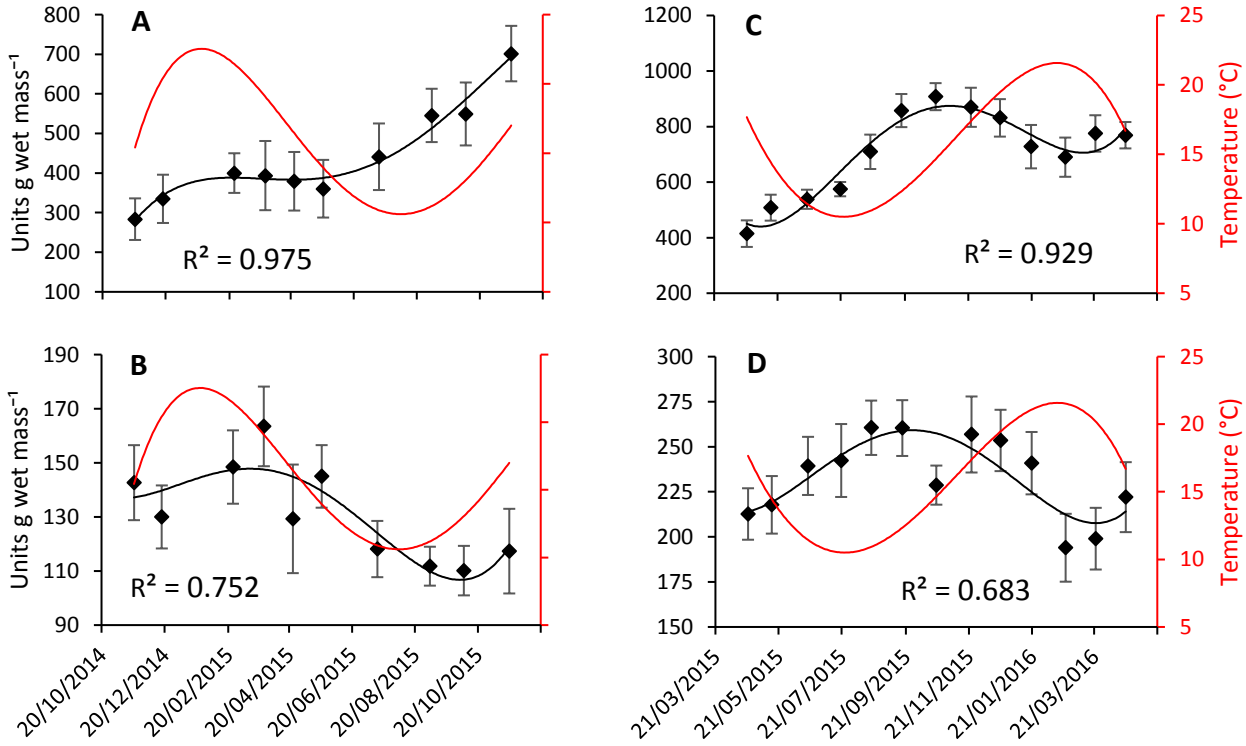

Figure 5.6. Pyruvate kinase (PK) activity in snapper (A – white muscle, B – red muscle) and YEM tissues (C – white muscle, D – red muscle).

5.3.1.5.2 YEM

Similar to what was observed for snapper, sea-water temperature did not show significant correlation with PK activity in YEM WM (Table 5.2, Fig. 5.6C). On the other hand, PK activity in RM, as already detected for LDH RM and HOAD WM, was negatively correlated with temperature (Table 5.2, Fig. 5.6D). In terms of inter-tissue association, there was no statistically detected correlation in annual behaviour of PK activity between white and red muscle (predicted – $R = 0.482$, $R^2 = 0.232$, $F_{1, 12} = 3.33$, $p = 0.095$), although, analogous to HOAD activities in the same muscle tissues, annual trajectories appeared to follow comparable routes.

5.3.1.6 Trypsin (TRY) and chymotrypsin (CHY) – correlation with temperature and between enzymes

5.3.1.6.1 Snapper

None of serine proteases (TRY and CHY) extracted from snapper pyloric caeca demonstrated any resemblance with annual sea-water temperature fluctuation (Fig. 5.7A, B). Accordingly, there were no correlations observed (Table 5.1). The dynamics of TRY and CHY were distinctly different to any other enzyme activity patterns observed in this study. This also contributed to weak explanatory power of 4th polynomial smoothening function explaining the data (i.e. $R^2 = 0.297$ and $R^2 = 0.151$ for TRY and CHY respectively). However, since the physiological functions of the two enzymes are mechanistically linked their annular trajectories were also highly positively correlated (raw – $R = 0.780$, $R^2 = 0.609$, $F_{1, 9} = 12.46$, $p = 0.008$).

5.3.1.6.2 YEM

In contrast to snapper serine proteases, both YEM TRY and CHY were correlated (negatively) with temperature (Table 5.2, Fig. 5.7C, D), and the 4th polynomial model well explained activity data (i.e. $R^2 = 0.791$ and $R^2 = 0.697$ for TRY and CHY respectively). As expected, enzymes were also positively correlated between each other (raw – $R = 0.643$, $R^2 = 0.413$, $F_{1, 12} = 7.75$, $p = 0.046$, predicted – $R = 0.900$, $R^2 = 0.806$, $F_{1, 12} = 45.79$, $p < 0.001$).

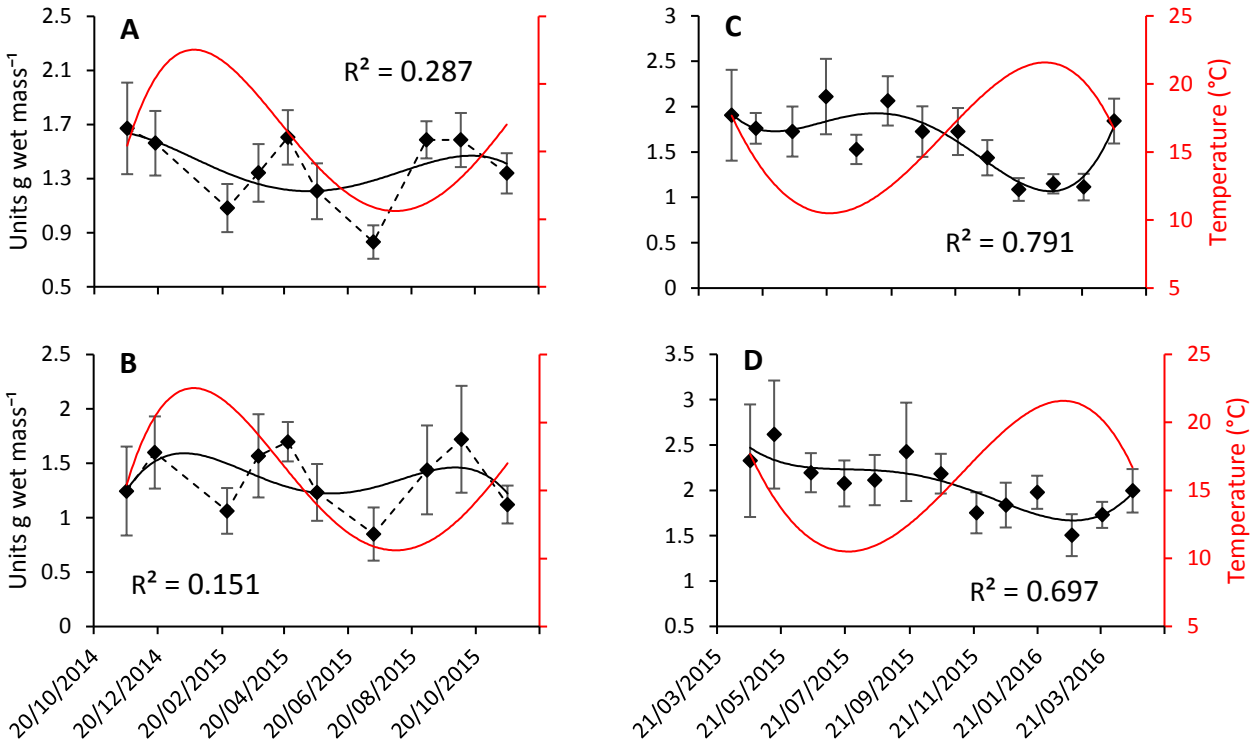

Figure 5.7. Enzymatic activity (black curve and black symbols) superimposed with sea-water temperature curve (red), in snapper (A – trypsin, B – chymotrypsin) and YEM pyloric caeca (C – trypsin, D – chymotrypsin). Dashed black lines in A and B that connect data points are placed to aid illustrating the annual enzyme dynamics since 4th polynomial function did not well explain the fitted data, as represented with associated R^2s.

5.3.2 Snapper and YEM tissue metabolites – effect of temperature

5.3.2.1 Lactate – correlation with temperature and between tissues

5.3.2.1.1 Snapper

Lactate concentration in snapper, in the three selected tissues, over one calendar year oscillated substantially, and in the case of RM and liver the dynamics of the oscillation was highly and positively correlated with sea-water temperature (Table 5.1, Fig. 5.8A, B, C). Therefore, the only significant lactate association amongst the three tissues was also observed between RM and liver (predicted – $R = 0.761$, $R^2 = 0.579$, $F_{1, 9} = 10.99$, $p = 0.011$).

5.3.2.1.2 YEM

YEM lactate content in RM did not correlate with temperature; however, in WM and liver a positive correlation for both, raw and predicted data, was evident (Table 5.2, Fig. 5.9D, F). Nevertheless, the lactate trajectories did not result in significant correlation between any of the tested tissues.

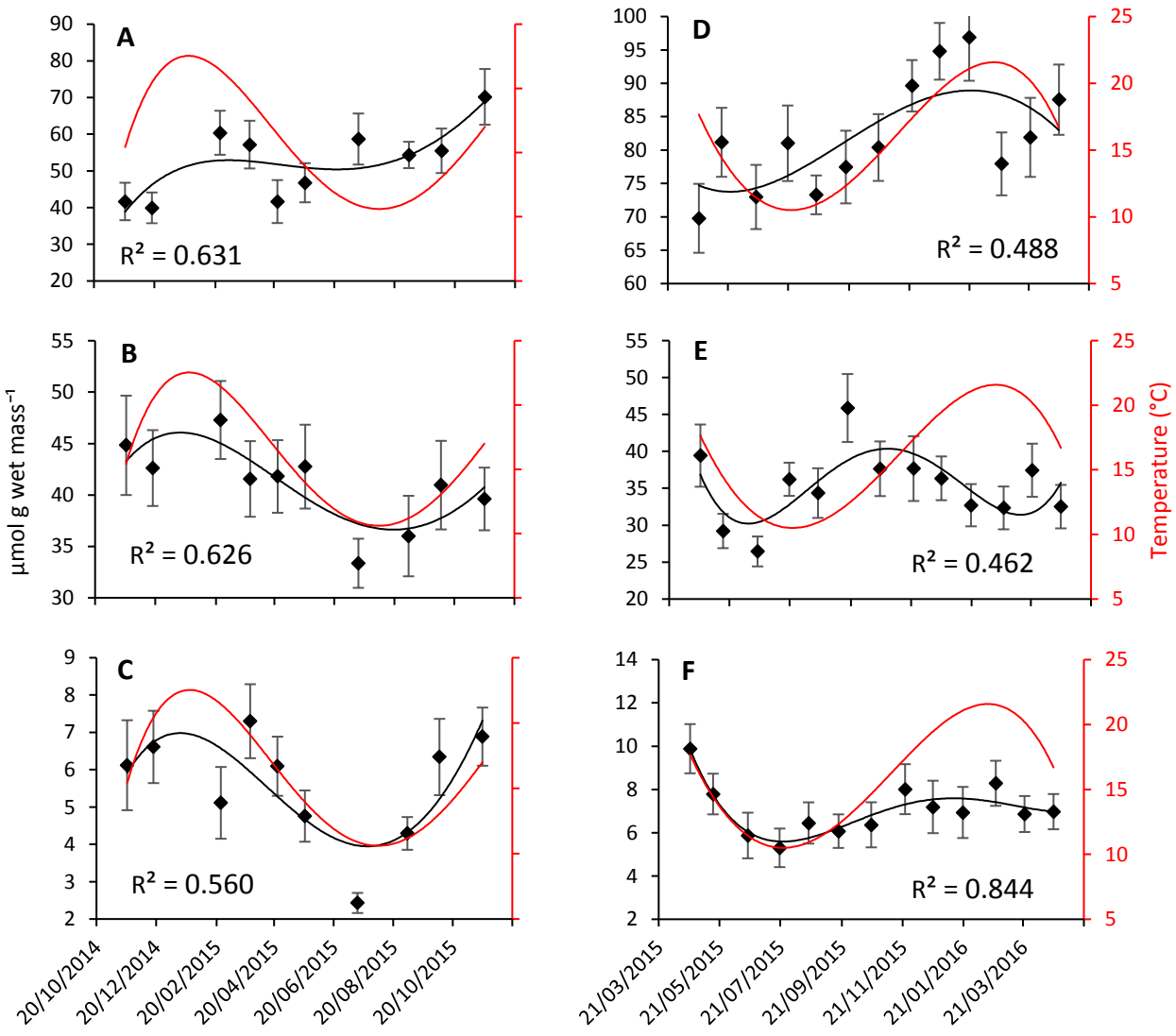

Figure 5.8. Lactate concentration in snapper (A – white muscle, B – red muscle, C – liver) and YEM tissues (D – white muscle, E – red muscle, F – liver).

5.3.2.2 Glucose – correlation with temperature and between tissues

5.3.2.2.1 Snapper

Snapper glucose in all three tissues of interest appeared to be related with the annual change in sea-water temperature (Fig. 5.9A, B, C). In WM the lag time in response to temperature change was evident, therefore glucose data were moved 2 months backwards on the annual temperature scale which assisted in revealing a strong positive correlation (Table 5.1). Annual glucose dynamics in RM and liver demonstrated the principle observed for lactate concentrations where the same type of correlation with temperature was found in both tissues; however, positive for lactate and negative for glucose (Fig. 5.9B, C). Consequently, a positive correlation between RM and liver glucose concentrations was also evident (raw – $R = 0.684$, $R^2 = 0.468$, $F_{1, 9} = 7.05$, $p = 0.029$, predicted – $R = 0.816$, $R^2 = 0.666$, $F_{1, 9} = 15.96$, $p = 0.004$).

5.3.2.2.2 YEM

Glucose concentration in YEM was only found to significantly correlate (negatively) with sea-water temperature in WM (Fig. 5.9D, Table 5.2). While in RM and liver, annual glucose concentration behaviour did not show a close resemblance with annual temperature dynamics; however, their somewhat similar trajectories resulted in positive correlation when data from the two tissues were regressed against each other (raw – $R = 0.620$, $R^2 = 0.385$, $F_{1, 12} = 6.88$, $p = 0.024$, predicted – $R = 0.553$, $R^2 = 0.306$, $F_{1, 12} = 4.85$, $p = 0.049$; Fig. 5.9E, F).

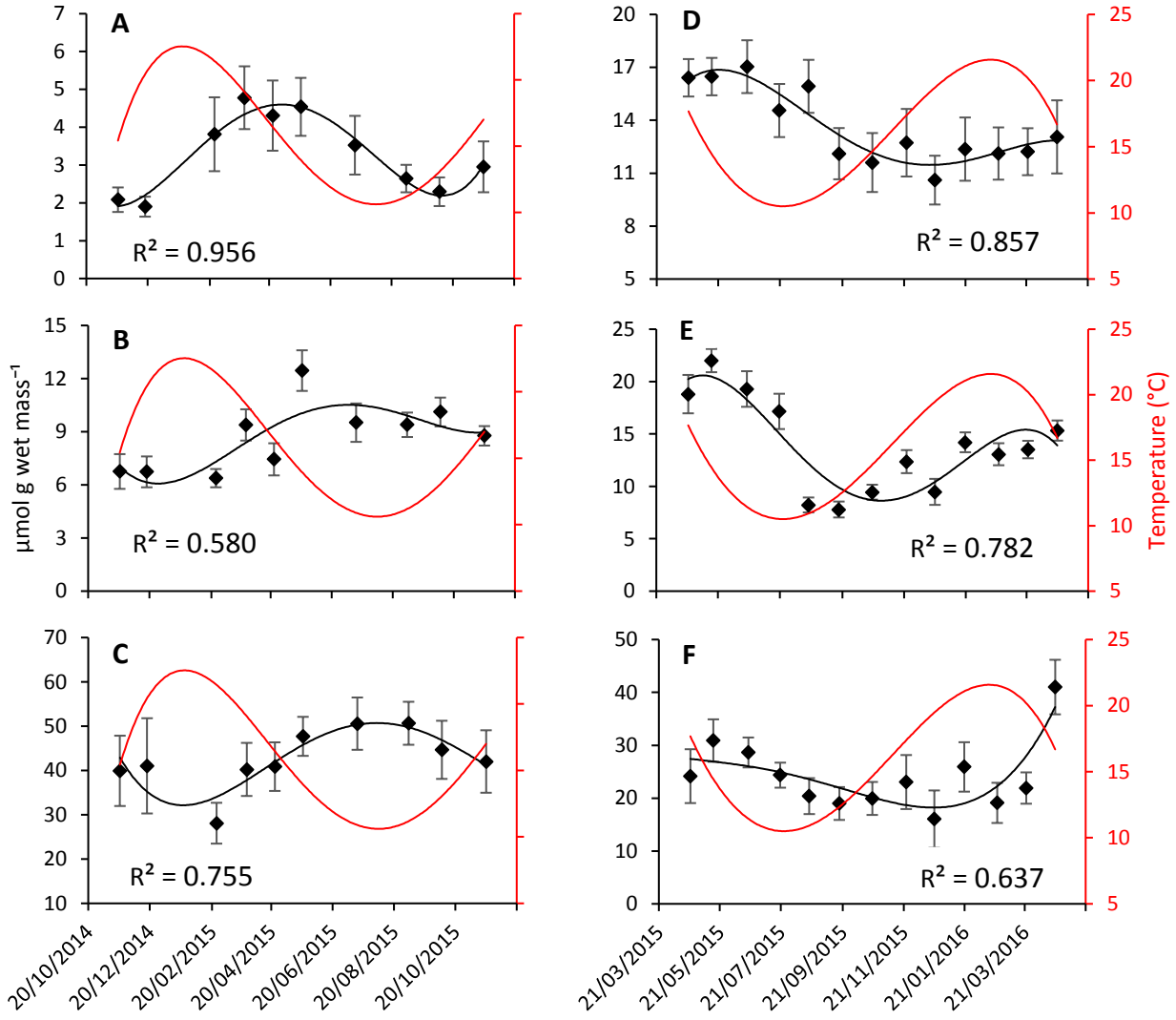

Figure 5.9. Glucose concentration in snapper (A – white muscle, B – red muscle, C – liver) and YEM tissues (D – white muscle, E – red muscle, F – liver).

5.3.2.3 Glycogen – correlation with temperature and between tissues

5.3.2.3.1 Snapper

Glycogen stores found in all three selected snapper tissues showed a significant correlation with sea-water temperature (Table 5.1). Nevertheless, associations were not direct, but rather in WM and liver 2-month and in RM 1-month lag time correlations were present. Besides, graphical presentation of data indicated that all three tissues had somewhat matching annual dynamics (Fig. 5.10A, B, C). This

was confirmed with demonstration of moderate to stronger correlations when data were regressed amongst the tissues. WM exhibited a positive correlation with both RM and liver (with RM – raw – $R = 0.653$, $R^2 = 0.426$, $F_{1, 9} = 5.94$, $p = 0.041$, predicted – $R = 0.675$, $R^2 = 0.456$, $F_{1, 9} = 6.71$, $p = 0.032$; with liver – raw – $R = 0.718$, $R^2 = 0.515$, $F_{1, 9} = 8.49$, $p = 0.019$, predicted – $R = 0.903$, $R^2 = 0.816$, $F_{1, 9} = 35.45$, $p < 0.001$). However, even though the correlation between RM and liver was moderate, it was not enough to be significant (predicted - $R = 0.593$, $R^2 = 0.351$, $F_{1, 9} = 4.33$, $p = 0.070$).

5.3.2.3.2 YEM

YEM glycogen in all three tested tissues did not correlate with temperature (Table 5.2, Fig. 5.10D, E, F). On the other hand, YEM glycogen was the only metabolite that showed annual dynamics in agreement across all three tissues, since a strong positive correlation was found amongst them (WM and RM, raw – $R = 0.990$, $R^2 = 0.981$, $F_{1, 12} = 560.89$, $p < 0.001$, WM and liver, raw – $R = 0.806$, $R^2 = 0.649$, $F_{1, 12} = 20.34$, $p < 0.001$, RM and liver, raw – $R = 0.850$, $R^2 = 0.722$, $F_{1, 12} = 28.61$, $p < 0.001$). From this, it became obvious that glycogen exhibited a distinct relationship amongst the three test tissues, which could be well explained with the linear model for the relationship between WM and RM (Fig. 5.11A), and with the natural logarithmic model for WM – liver (Fig. 5.11B) and RM – liver relationships (Fig. 5.11C).

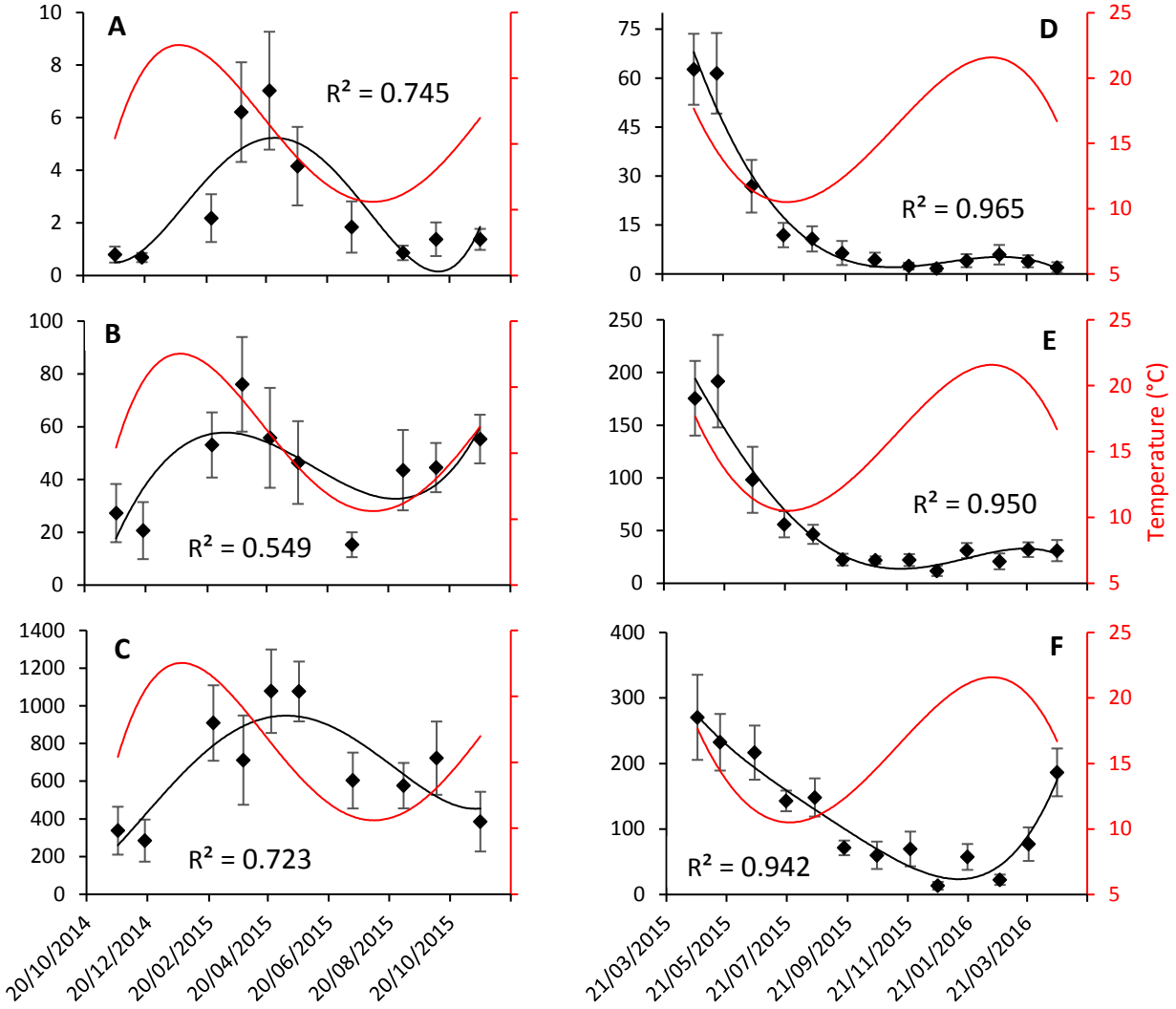

Figure 5.10. Glycogen concentration in snapper (A – white muscle, B – red muscle, C – liver) and YEM tissues (D – white muscle, E – red muscle, F – liver).

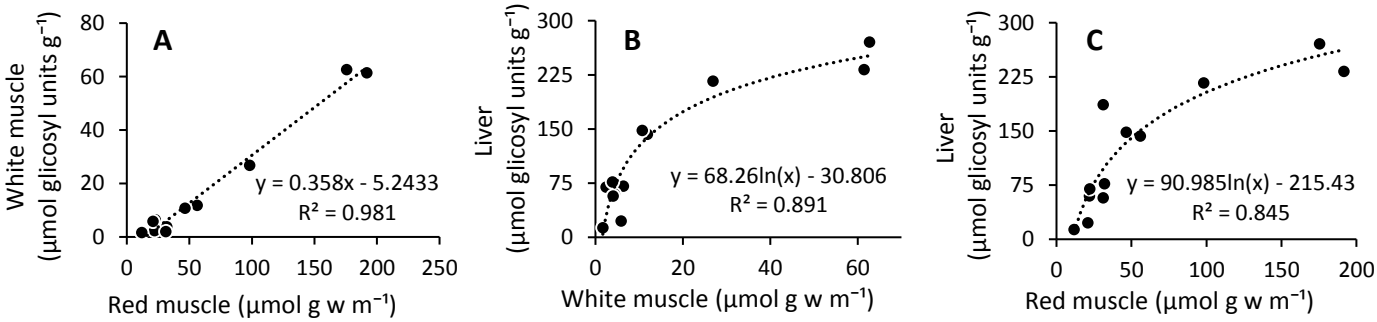

Figure 5.11. Relationship of glycogen stores expressed in glucosyl units gram wet mass^{-1} amongst three tissues in YEM, A – red and white muscle, with linear regression equation and R^2; B – white muscle and liver, with natural logarithmic equation and R^2; and C – red muscle and liver with natural logarithmic equation and R^2.

5.3.3 Enzymatic ratios – relative levels of aerobic and anaerobic enzymes

5.3.3.1 Enzymatic ratios – correlation with temperature

Enzyme ratios demonstrated different patterns in the test species when they were correlated with annual sea-water temperature. In the case of snapper, prevailing associations were positive (indicating snapper tendency to thermally conform) and lag-positive (with the peak in late autumn, indicating metabolic synchronisation for the winter preparation), whereas in YEM, most correlations had a negative direction, suggesting a greater YEM capacity for temperature compensation. Annual mean values with associated standard deviations of selected enzymatic ratios together with their correlations with sea-water temperature and growth parameters (i.e. specific mass growth rates – SMGR, and absolute cumulative mass gain – ACMG) were summarised for both species in Table 5.4.

5.3.3.2 Intra-specific (inter-tissue) and inter-specific annual mean enzymatic ratios comparison

Two-way ANOVA on annual mean CS/CCO ratio data, after pairwise multiple comparison by Holm-Sidak method, showed that the only difference in snapper was found between RM and intestines ($t = 2.769$, $p = 0.022$, Fig. 5.12). At the same time, in YEM, all tissue combinations were shown to be significantly different, where the lowest annual ratio was found in intestine and the highest in RM (RM – intestines $t = 13.246$, $p < 0.001$; RM – WM $t = 7.451$, $p = < 0.001$; WM – intestine $t = 5.795$, $p < 0.001$; Table 5.4). Inter-specifically, the ratio was statistically the same in intestines, the most different in RM and less intense but still evidently different in WM, with YEM overall demonstrating greater activities of the Krebs cycle relative to the electron transport chain (Table 5.3, Fig. 5.12).

HOAD/CS ratio differed between two muscle tissues in both species, as two-way ANOVA on square root transformed data revealed ($t = 3.825$, $p < 0.001$ and $t = 8.312$, $p < 0.001$ for snapper and YEM respectively, Fig. 5.13A). However, mean ratios between corresponding tissues of two species were statistically the same (Table 5.3, Fig. 5.13A), whereas, HOAD/CCO ratio appeared to be different for both comparisons, intra-specifically (i.e. snapper WM – RM $t = 2.532$, $p = 0.015$; YEM WM – RM $t = 15.793$, $p < 0.001$) and inter-specifically (Table 5.3, Fig. 5.13B). More importantly, the effect of different tissues on the calculated ratio depended on species, which was indicated with significant interaction between factors tissue and species in the ANOVA model ($F_{1, 45} = 72.429$, $p < 0.001$). LDH/CS ratio was ~70 times higher in WM compared with RM in both species (Fig. 5.14A, B). A difference was also found for both muscle tissues when compared between species (Table 5.3, Fig.

5.14). PK/LDH ratio did not differ between muscle tissues intra-specifically (Fig. 5.15). However, YEM ratio was higher in both white and red muscle (Table 5.3).

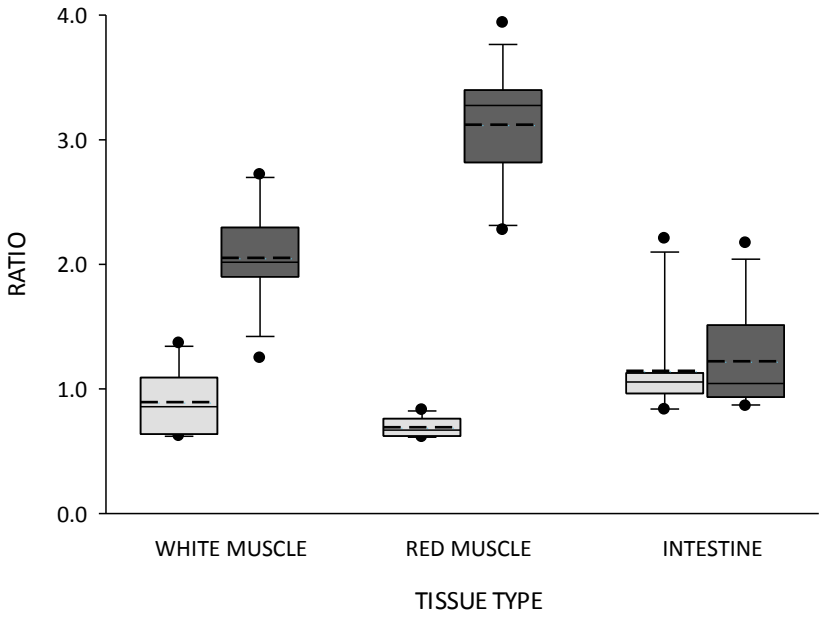

Figure 5.12. Box plot presentation of CS/CCO enzymatic ratio based on annual mean enzyme activity for three different tissue types (white muscle, red muscle and intestines) for snapper (light grey boxes) and YEM (dark grey boxes). The ends of the boxes define the 25th and 75th percentiles, with a middle line at the median and error bars defining the 10th and 90th percentiles. Black dots are data points outside of the 10th/90th percentiles envelop. Dashed lines within boxes represent mean values.

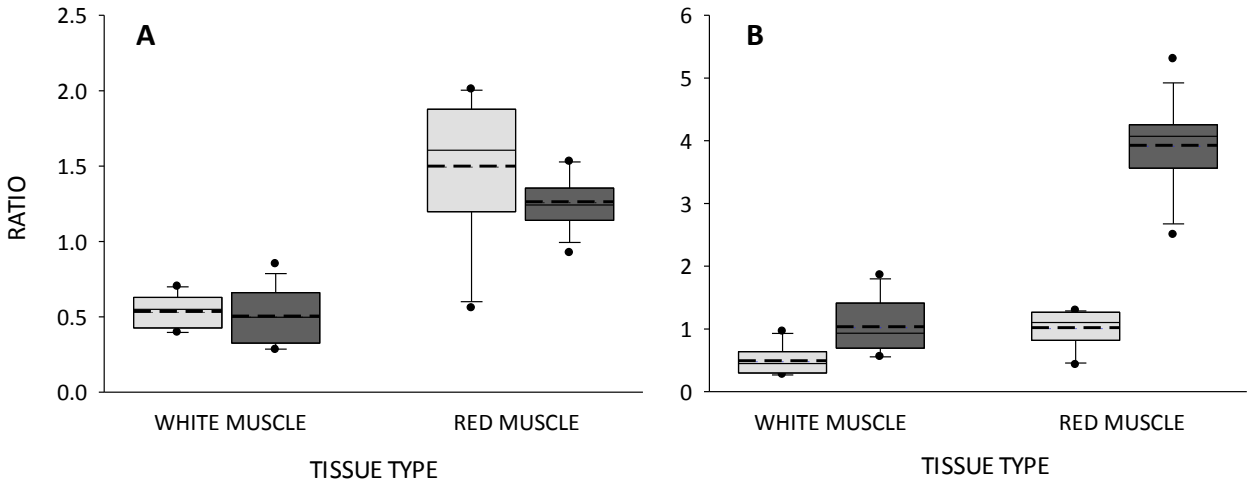

Figure 5.13. Box plot presentation of HOAD/CS (A) and (B) HOAD/CCO enzymatic ratios based on annual mean enzyme activity for two muscle tissue (white and red muscle) for snapper (light grey boxes) and YEM (dark grey boxes). The rest of the figure caption is the same as for Fig. 5. 12.

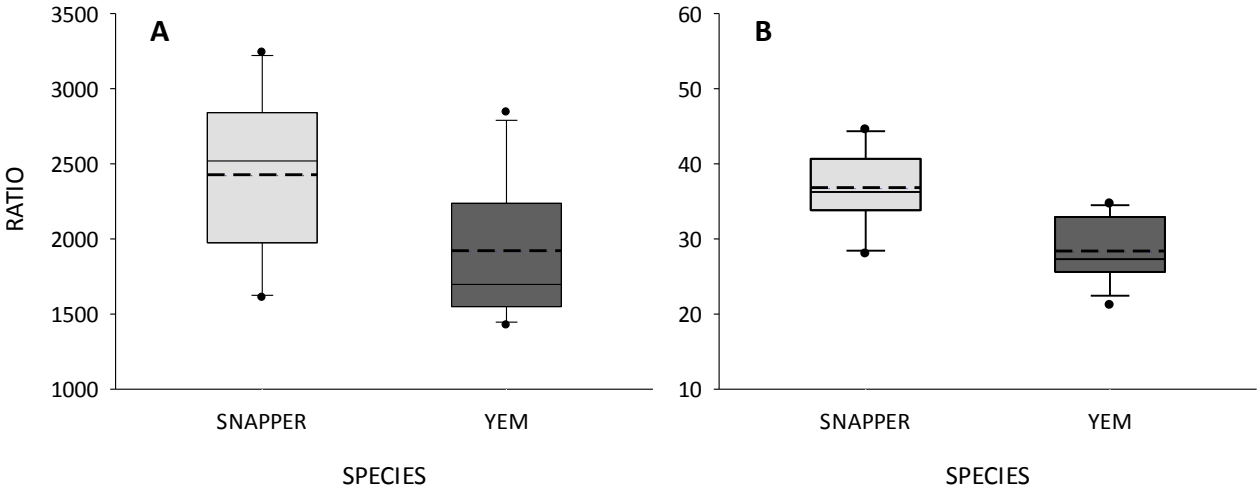

Figure 5.14. Box plot presentation of LDH/CS enzymatic ratio in white (A) and red muscle (B) based on annual mean enzyme activity for snapper (light grey boxes) and YEM (dark grey boxes). The rest of the figure caption is the same as for Fig. 5. 12.

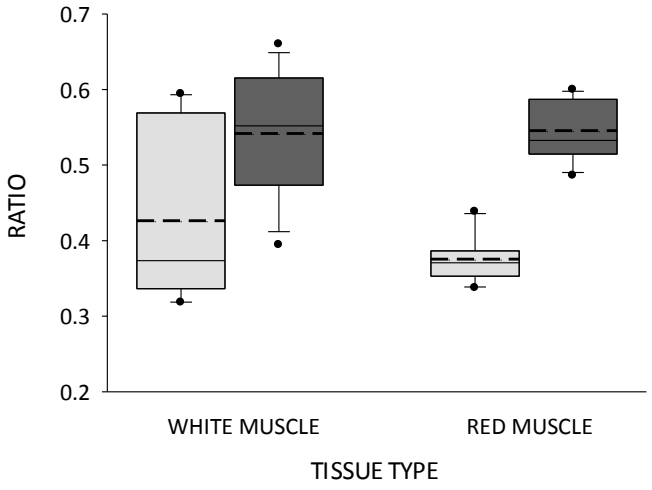

Figure 5.15. Box plot presentation of PK/LDH enzymatic ratio based on annual mean enzyme activity for two muscle tissue (white and red muscle) for snapper (light grey boxes) and YEM (dark grey boxes). The rest of the figure caption is the same as for Fig. 5. 12.

Table 5.1. Snapper enzymatic activity and tissue metabolite concentrations and their correlations with sea-water temperature (TEMP) and growth parameters (i.e. specific mass growth rates – SMGR, and absolute cumulative mass gain – ACMG) accompanied with corresponding coefficient of correlations (COEFF), p-values and type of correlation (TYPE: positive – POS; negative – NEG; lag time positive – LAG P) in white muscle (WM), red muscle (RM), intestines (IN), liver and pyloric caeca (PC). CCO – cytochrome c oxidase, CS – citrate synthase, HOAD – 3hydroxyacyl-CoA dehydrogenase, LDH – lactate dehydrogenase, PK – pyruvate kinase, TRY – trypsin, CHY – chymotrypsin.

SNAPPER		**CORRELATIONS**								
		SEA-WATER TEMP			**SMGR**			**ACMG**		
ENZYMES	TISSUE	COEFF	P-VALUE	TYPE	COEFF	P-VALUE	TYPE	COEFF	P-VALUE	TYPE
CCO	WM	**0.73**	**0.016**	**LAG P**	0.004	0.991	NS	**0.76**	**0.011**	**NEG**
	RM	0.32	0.375	NS	0.33	0.346	NS	0.45	0.195	NS
	IN	0.08	0.824	NS	0.16	0.668	NS	**0.64**	**0.047**	**NEG**
CS	WM	**0.73**	**0.016**	**POS**	**0.69**	**0.027**	**POS**	**0.78**	**0.007**	**NEG**
	RM	0.06	0.862	NS	0	0.959	NS	**0.82**	**0.004**	**NEG**
	IN	**0.76**	**0.008**	**POS**	**0.72**	**0.021**	**POS**	**0.9**	**< 0.001**	**NEG**
HOAD	WM	0.57	0.086	NS	**0.71**	**0.021**	**POS**	0.31	0.389	NS
	RM	0.01	0.968	NS	0.11	0.789	NS	**0.82**	**0.004**	**POS**
	LIVER	0.54	0.109	NS	0.45	0.197	NS	**0.85**	**0.002**	**NEG**
LDH	WM	0.26	0.471	NS	0.32	0.361	NS	**0.64**	**0.046**	**POS**
	RM	0.53	0.112	NS	**0.65**	**0.041**	**POS**	**0.65**	**0.042**	**NEG**
PK	WM	0.28	0.432	NS	**0.83**	**0.003**	**POS**	**0.73**	**0.018**	**POS**
	RM	**0.69**	**0.027**	**POS**	**0.83**	**0.003**	**POS**	0.49	0.155	NS
TRY	PC	0.11	0.774	NS	0.07	0.847	NS	**0.67**	**0.035**	**NEG**
CHY	PC	0.38	0.278	NS	0.35	0.327	NS	0.35	0.324	NS
METABOLITES										
LACTATE	WM	0.06	0.862	NS	0.15	0.67	NS	**0.79**	**0.006**	**POS**
	RM	**0.92**	**< 0.001**	**POS**	**0.93**	**< 0.001**	**POS**	**0.73**	**0.017**	**NEG**
	LIVER	**0.81**	**0.004**	**POS**	**0.69**	**0.027**	**POS**	0.37	0.293	NS
GLUCOSE	WM	**0.84**	**0.002**	**LAG P**	0.36	0.308	NS	0.44	0.204	NS
	RM	**0.77**	**0.009**	**NEG**	**0.7**	**0.024**	**NEG**	**0.88**	**< 0.001**	**POS**
	LIVER	**0.98**	**< 0.001**	**NEG**	**0.97**	**< 0.001**	**NEG**	0.53	0.111	NS
GLYCOGEN	WM	**0.94**	**< 0.001**	**LAG P**	0.42	0.23	NS	0.31	0.393	NS
	RM	**0.62**	**0.056**	**LAG P**	0.52	0.155	NS	0.51	0.133	NS
	LIVER	**0.88**	**< 0.001**	**LAG P**	0.16	0.661	NS	0.5	0.142	NS

Table 5.2. YEM enzymatic activity and tissue metabolite concentrations. The rest of the Table caption is the same as for Table 5.1.

YEM		**CORRELATIONS**								
		SEA-WATER TEMP			**SMGR**			**ACMG**		
ENZYMES	TISSUE	COEFF	P-VALUE	TYPE	COEFF	P-VALUE	TYPE	COEFF	P-VALUE	TYPE
CCO	WM	0.37	0.215	NS	**0.55**	**0.054**	**NEG**	0.53	0.064	NS
	RM	0.14	0.658	NS	**0.67**	**0.012**	**POS**	0.18	0.565	NS
	IN	**0.86**	**< 0.001**	**POS**	0.19	0.534	NS	**0.59**	**0.034**	**POS**
CS	WM	0.31	0.304	NS	**0.84**	**< 0.001**	**NEG**	**0.65**	**0.016**	**POS**
	RM	0.4	0.177	NS	**0.73**	**0.005**	**NEG**	0.48	0.095	NS
	IN	0.29	0.337	NS	0.14	0.646	NS	0.09	0.758	NS
HOAD	WM	**0.64**	**0.018**	**NEG**	0.24	0.432	NS	**0.55**	**0.051**	**NEG**
	RM	0.11	0.733	NS	**0.92**	**< 0.001**	**NEG**	0.44	0.129	NS
	LIVER	0.44	0.128	NS	**0.56**	**0.046**	**POS**	0.03	0.911	NS
LDH	WM	0.33	0.277	NS	**0.89**	**< 0.001**	**NEG**	**0.75**	**0.003**	**POS**
	RM	**0.96**	**< 0.001**	**NEG**	0.16	0.594	NS	**0.62**	**0.023**	**NEG**
PK	WM	0.21	0.492	NS	**0.85**	**< 0.001**	**NEG**	0.53	0.063	NS
	RM	**0.62**	**0.025**	**NS**	0.43	0.145	NS	0.45	0.119	NS
TRY	PC	**0.83**	**< 0.001**	**NEG**	0.24	0.417	NS	**0.64**	**0.017**	**NEG**
CHY	PC	**0.74**	**0.004**	**NEG**	**0.6**	**0.03**	**POS**	**0.85**	**< 0.001**	**POS**
METABOLITES										
LACTATE	WM	**0.74**	**0.004**	**POS**	**0.61**	**0.027**	**NEG**	**0.75**	**0.003**	**POS**
	RM	0.01	0.985	NS	0.21	0.494	NS	0.07	0.812	NS
	LIVER	**0.64**	**0.019**	**POS**	**0.72**	**0.006**	**POS**	0.01	0.985	NS
GLUCOSE	WM	**0.6**	**0.032**	**NEG**	**0.7**	**0.008**	**POS**	**0.7**	**0.008**	**NEG**
	RM	0.06	0.852	NS	**0.79**	**0.001**	**POS**	0.3	0.328	NS
	LIVER	0.14	0.647	NS	0.21	0.499	NS	0.26	0.389	NS
GLYCOGEN	WM	0.17	0.569	NS	**0.91**	**< 0.001**	**POS**	**0.65**	**0.017**	**NEG**
	RM	0.23	0.456	NS	**0.91**	**< 0.001**	**POS**	**0.62**	**0.024**	**NEG**
	LIVER	0.44	0.131	NS	**0.63**	**0.021**	**POS**	0.49	0.089	NS

Table 5.3. Snapper versus YEM annual mean enzymatic activity ratios comparison carried out with two-way ANOVA with associated t-test statistics (*t*) and p-values (*p*). Bold and italic represent the data with significant ($p \leq 0.05$) and non-significant ($p > 0.05$) inter-specific differences respectively. Abbreviations are the same as for Table 5.1.

SNAPPER VS. YEM

	WM		RM		IN	
RATIO	*t*	*p*	*t*	*p*	*t*	*p*
CS/CCO	**7.54**	**< 0.001**	**15.79**	**< 0.001**	*0.50*	*0.620*
HOAD/CS	*0.51*	*0.617*	*1.61*	*0.116*		
HOAD/CCO	**2.76**	**0.008**	**14.8**	**< 0.001**		
LDH/CS	**2.41**	**0.025**	**4.46**	**< 0.001**		
PK/LDH	**2.9**	**0.009**	**11.34**	**< 0.001**		

Table 5.4. Snapper and YEM annual mean values (MEAN) of enzymatic ratios with associated standard deviation (S.D.) and correlations with sea-water temperature and growth parameters accompanied with corresponding coefficient of correlations, p-values and type of correlation. Abbreviations are the same as per Table 5.1.

SNAPPER			**SEA-WATER TEMP**			**SMGR**			**ACMG**		
RATIO	TISSUE	MEAN ± S.D.	COEFF	P-VALUE	TYPE	COEFF	P-VALUE	TYPE	COEFF	P-VALUE	TYPE
CS/CCO	WM	0.89 ± 0.24	**0.76**	**0.011**	**LAG P**	0.41	0.237	NS	0.48	0.156	NS
	RM	0.69 ± 0.08	0.54	0.111	NS	0.48	0.163	NS	**0.97**	**< 0.001**	**NEG**
	IN	1.15 ± 0.37	**0.72**	**0.019**	**POS**	**0.76**	**0.01**	**POS**	0.08	0.819	NS
HOAD/CS	WM	0.54 ± 0.11	**0.93**	**< 0.001**	**LAG P**	0.14	0.692	NS	0.31	0.383	NS
	RM	1.50 ± 0.43	0.02	0.962	NS	0.07	0.845	NS	**0.81**	**0.004**	**POS**
HOAD/CCO	WM	0.49 ± 0.21	**0.93**	**< 0.001**	**LAG P**	0.32	0.368	NS	0.39	0.262	NS
	RM	1.02 ± 0.28	0.17	0.641	NS	0.22	0.535	NS	**0.75**	**0.013**	**POS**
LDH/CS	WM	2427.97 ±509.45	0.42	0.224	NS	0.33	0.345	NS	**0.82**	**0.004**	**POS**
	RM	36.86 ± 4.65	0.36	0.311	NS	0.49	0.155	NS	0.45	0.196	NS
PK/LDH	WM	0.43 ± 0.11	0.42	0.228	NS	0.59	0.075	NS	0.57	0.083	NS
	RM	0.38 ± 0.03	**0.7**	**0.024**	**POS**	**0.83**	**0.003**	**POS**	0	0.998	NS
YEM											
CS/CCO	WM	2.05 ± 0.37	0.31	0.301	NS	0.46	0.11	NS	0.07	0.828	NS
	RM	3.12 ± 0.44	0.26	0.394	NS	**0.84**	**< 0.001**	**NEG**	0.44	0.131	NS
	IN	1.22 ± 0.40	**0.94**	**< 0.001**	**NEG**	0.11	0.712	NS	**0.64**	**0.019**	**NEG**
HOAD/CS	WM	0.51 ± 0.17	**0.67**	**0.013**	**NEG**	**0.67**	**0.012**	**POS**	**0.92**	**< 0.001**	**NEG**
	RM	1.26 ± 0.16	**0.65**	**0.015**	**NEG**	**0.65**	**0.016**	**NEG**	0.13	0.682	NS
HOAD/CCO	WM	1.04 ± 0.42	**0.88**	**< 0.001**	**NEG**	0.23	0.449	NS	**0.82**	**< 0.001**	**NEG**
	RM	3.93 ± 0.66	0.12	0.704	NS	**0.98**	**< 0.001**	**NEG**	0.41	0.16	NS
LDH/CS	WM	1923.12 ± 448.35	0.26	0.382	NS	**0.87**	**< 0.001**	**POS**	**0.63**	**0.022**	**NEG**
	RM	28.40 ± 4.02	**0.68**	**0.01**	**NEG**	**0.56**	**0.046**	**POS**	**0.66**	**0.015**	**NEG**
PK/LDH	WM	0.54 ± 0.08	0.13	0.675	NS	**0.63**	**0.021**	**NEG**	0.4	0.172	NS
	RM	0.55 ± 0.04	0.09	0.773	NS	0.45	0.122	NS	0.16	0.598	NS

5.4 Discussion

5.4.1 General observation

Based on annual averages, as expected, in white muscle (WM) activities of glycolytic enzymes were distinctly higher than in red muscle (RM) (i.e.~3 times for both lactate dehydrogenase, LDH, and pyruvate kinase, PK, in both species), but the enzymes of oxidative/aerobic pathways were more than an order of magnitude more active in RM than in WM (i.e. ~30 times for cytochrome *c* oxidase, CCO, in snapper, ~15 times CCO in YEM; ~20 times for citrate synthase, CS, and ~60 times for 3-hydroxyacyl-CoA dehydrogenase, HOAD, in both species) indicating the importance of oxidative energy generation with a preference for lipid oxidation in RM and utilisation of anaerobic glycolysis as a main energy provider for WM. Interspecifically, biochemical analysis revealed markedly different profiles of the two test species in terms of response to change in seasonal environmental parameters to energy demand for growth and survival. When characteristics of key metabolic pathways were observed in terms of metabolites, snapper demonstrated that the most prominent feature was related to the event of boosting fish condition prior to winter, similar to the pattern observed for enzyme activities and their ratios. YEM showed high importance of metabolic involvement towards storing energy; however, this may not be related to seasons but is likely a reflection of the *ad libitum* feeding regime employed in annual growth trials.

5.4.2 Biochemical profiles of test species and their relation to growth

5.4.2.1 Muscle aerobic capacity and growth

Enzyme activities in fish have been shown to vary according to changes in many environmental parameters, though some have been shown to have more pronounced impacts on tissue metabolic capacities, such as water temperature (Buckley, 1982; Guderley, 2004), food availability (Moon and Johnston, 1980, Goolish and Adelman, 1987) and growth/maturation (Campana and Hurley, 1989). From a biochemical perspective a commonly reported link with growth traits are activities of glycolytic and mitochondrial enzymes in muscle and mitochondrial enzymes in intestinal tissue (Mathers et al., 1992; Foster et al., 1993; Pelletier et al., 1993b; Pelletier et al., 1995; Blier et al., 1997; Couture et al., 1998; Dutil et al., 1998; Gauthier et al., 2008). However, there is debate about which enzymatic group better represents growth performance. For instance, Mathers et al. (1992) reported significant correlation between CS and CCO activity in WM and growth rates (GR) of saithe (*Pollachius*

virens). They argued that activity of enzymes associated with aerobic energy production in WM can be employed for estimating GR, because protein synthesis as a representation of growth requires energy, hence for GR to increase, aerobic capacity of tissues the most engaged in growth (i.e. WM, Houlihan et al. 1988) should also increase (Goolish and Adelman, 1987). By contrast, Pelletier et al. (1995) did not find this link and they concluded that Atlantic cod do not increase the aerobic capacity of WM to specifically accompany change in GR. Pelletier et al. (1995) further argued that the connection between aerobic enzymes and GR in other studies were likely manifestations of the general response of protein concentration and not the adjustment of the tissue aerobic pathways to meet requirements of protein synthesis. Furthermore, Blier et al. (1997) demonstrated that at least for Atlantic cod the direct relationship cannot stand since aerobic capacity of cod WM for ATP production was found to be ~10 times higher than the energy required for protein synthesis even in the low mitochondrial density tissue such as WM (Johnston and Moon, 1981).

Nevertheless, a muscle mitochondrial enzyme/GR relationship has been reported in subsequent studies (e.g. Couture et al., 1998; Gauthier et al., 2008) including the present one where positive CCO correlation with GR was found in YEM, though in this case in RM, and CS activity was positively associated with GR in snapper WM, but atypically – negatively in both YEM muscle tissues. A simple explanation for the uncommon relationship between CS activity and GR in YEM can be proposed by a much stronger positive correlation between enzymatic activity and visceral lipid index (relative visceral mass, VLI). However, it is unlikely that increased visceral fat stores required increased aerobic activity of WM, in which case WM aerobic machinery would be actively involved in the process. It has been suggested that muscle proteins (including enzymes) are not only functionally important but that they also represent important energy reserves (Couture et al., 1998) and protein stores (Houlihan et al., 1988). Therefore, a more plausible scenario would be that favourable conditions (i.e. predominantly unlimited food supply) allowed YEM to fill up energy stores until the optimal levels (e.g. ~ 15% body mass for visceral fat deposits) are reached, and that the build-up was simultaneous across all preferable energy store types, which for YEM appeared to be lipids and proteins. Furthermore, that could also mean that oxidative capacity of YEM WM is even at its lowest levels sufficient to support protein synthesis during highest GR, the scenario also observed for Atlantic cod, saithe (Blier et al., 1997) and Arctic charr, *Salvelinus fantinalis* (Blier and Lemieux, 2001). However, in YEM RM, since CCO activity was positively related with GR, requirements for growth was suggested, and accordingly CCO activity was modified, possibly by mitochondrial membrane alteration and/or adjustments in cristae density (Guderley, 2004).

The different responses of CCO and CS in the same tissue to the change in GR as observed in other and the present study are not unexpected. CS depends on mitochondrial volume since it is a soluble matrix enzyme, whereas CCO as it is embodied into the inner mitochondrial membrane reflects any changes in its total surface area (i.e. cristae density) (Pelletier et al., 1995). Therefore, their activities

may correspond in concert with the change in the surface/volume ratio of mitochondria, due to alteration of their size/shape when adjusting to environmental conditions (i.e. temperature and food supply) (Pelletier et al., 1995; Guderley, 2004). In the present study the positive correlation between CCO and CS in both white and red muscle in snapper and in WM in YEM were observed, suggesting that in many cases their activities were linked either via their function or merely due to little or no change in mitochondrial properties as a result of change in growth performance.

5.4.2.2 Intestinal aerobic capacity and growth

The aerobic capacity of intestines is more generally accepted to be better linked to the GR of fish owing to its pivotal role in providing for ATP as a fuel for nutrient absorption and transport (Pelletier et al., 1994). This notion is supported by numerous studies where a positive relationship between aerobic activity and GR was observed (e.g. Pelletier et al., 1994; Couture et al., 1998; Dutil et al., 1998; this study). In Atlantic cod (Pelletier et al., 1994; Couture et al., 1998), a positive correlation between aerobic enzymes and GR together with unchanged ESI (entero-somatic index or relative intestinal mass) led the authors to conclude that the aerobic capacity in intestine may limit nutrient absorption and ultimately growth in adult cod. However, for other species, such as largemouth bass, *Micropterus salmoides* (Goolish and Adelman, 1987), a positive change in both ESI and intestinal aerobic activity with change in GR was observed. This scenario resembles findings in the present study, though only snapper had both CS activity and ESI positively correlated with GR. Furthermore, in snapper no CCO/GR relationship was observed, but rather a weak thermal compensation (the maintenance of physiological rate as the temperature changes; Clarke, 1983). In addition, CCO activity in snapper intestine appeared to be unvarying for the last ~2/3 of the study. All of this may suggest that, although capacity of the Krebs cycle may be fully exploited (an insight based on positive intestinal CS/GR relationship) but because the activity of the cycle was not followed by activity of electron transport chain (ETC) that the capacity of CCO and oxidative phosphorylation in intestinal wall, similarly as found in Atlantic cod (Blier et al., 1997), may be higher than the energy demand for processing any levels of feed intake and GR. Therefore, it appears that overall aerobic capacity of intestines is not limiting to GR in juvenile snapper.

On the other hand, YEM appeared to regulate the intestinal metabolic capacity by a combination of altering intestinal mass in accord with change in GR, partially by thermal compensatory response and partially by some other mechanisms since compensatory responses were not strong, especially for CCO. Again, there was not enough evidence to suggest that growth is limited by aerobic capacity of intestines in YEM.

5.4.2.3 Muscle glycolytic capacity and growth

In contrast to aerobic enzymes, glycolytic enzymes in WM often show more regular correlation with fish GR (Pelletier et al., 1993, 1994, 1995; Dutil et al., 1998; Isani et al., 2000; Rennie et al., 2005). The relationship, however, may not mean that activities of glycolytic enzymes are directly involved in the act of growth. Pelletier et al. (1995) demonstrated that in Atlantic cod the activity of glycolytic enzymes (LDH and PK) correlated with GR and that the relationship was independent of a change in overall muscle protein content and therefore it appeared that the increase in the enzymatic activity was due to their active involvement in the growth process and not just a consequence of increased protein production caused by augmented growth. However, it has been suggested that the link between glycolytic activity and growth is likely ecologically relevant and that fish with more rapid growth may have enhanced glycolytic capacity in WM as a main energy source supporting burst locomotion (Sullivan and Somero, 1983; Pelletier et al., 1993; Guderley et al., 1996), which in terms of growth is required for prey/food capture (Gauthier et al., 2008). Therefore, despite a positive relationship between glycolytic enzymes and GR it is unlikely that the enzymes are critical for protein synthesis (Pelletier et al., 1994), but rather it seems that GR regulates the WM glycolytic capacity (Dutil et al., 1998).

In addition, a few investigations reported a link between LDH and PK activity and fish condition indices (Guderley et al., 1996; Couture et al., 1998; Dutil et al., 1998; Gauthier et al., 2008). In the present study activities of glycolytic enzymes were related to both GR (YEM) and condition (snapper). The main findings in this regards for snapper was that LDH activity in WM strongly reflected fish condition due to its positive correlation with *Rm* (relative mass as an expression of condition) and VLI, while in YEM, both glycolytic WM enzymes (LDH and PK) demonstrated, as seen for oxidative enzymes, a pattern of negative correlation with GR. However, this relationship, again, may be just a by-product of a much stronger relationship of glycolytic enzymes with YEM VLI. Therefore, in snapper LDH activity in WM is predominantly an expression of energy status and reserves and not the response to change in GR. In similar fashion in YEM, despite the glycolytic activities being linked to GR the scenario is likely the same as explained for mitochondrial enzymes, an integral part of overall body energy reserves dynamics.

Strong correlation between LDH and VLI, as found in both test species is not unusual since VLI is a marker of fish energy status and levels of accumulated lipid stores (Nelson and Magnuson, 1992; Mackereth et al., 1998), and LDH apart from being the main catalyst in anaerobic glycolysis is also an indicator of muscle protein storage and energy status (Houlihan et al., 1988). According to this it is plausible that the link between the entire array of enzyme activities in YEM that demonstrated strong

positive correlation with VLI is a reflection of overall YEM nutritional/energy status including all body energy (lipids and proteins) and construction material (protein) stores. The stores' optimisation, therefore, may be a preferable physiological YEM activity to support fitness, survival and reproductive output, which in this study was possible through unlimited food supply.

5.4.2.4 Serine proteases capacity and growth

For some fish, such as Atlantic cod, it has been demonstrated that GR has a non-linear, plateauing relationship with food intake, which means that the capacity for growth is limited at a certain level of feed consumption and any further increase in feeding is not followed by increase in GR (Houlihan et al., 1988). Therefore, maximum GR may be limited at the point of digestion and nutrient transport (Lemieux et al., 1999), which rely on activity levels of digestive enzymes and the capacity for trans-epithelial transfer in the gastro-intestinal tract (Jobling, 1995). In particular, serine proteases, trypsin (TRY) and chymotrypsin (CHY), have been proposed as responsive and reliable biological markers for evaluating and comparing digestive efficiency and potential growth rates (Rungruangsak-Torrissen et al., 2006). Association between serine proteases, especially TRY, and GR as well as FCR (feed conversion efficiency) has been found in several fish species (e.g. Atlantic cod – Lemieux et al., 1999; Atlantic salmon – Sunde et al., 2001; Rungruangsak-Torrissen et al., 2006; striped bass, *Morone saxatilis* – Baragi and Lovell, 1986). The pattern of activity of TRY and CHY in response to GR that has been observed in salmonid studies (Sunde et al., 2001; Rungruangsak-Torrissen et al., 2006) indicates that TRY activity as well as TRY/CHY ratio increased when GR was enhanced, therefore it appears that TRY is important when growth is supported, while CHY seems to be imperative when growth in response to food deficiency or starvation is impaired and as a result TRY/CHY ratio is diminished.
Since TRY and CHY activities are functionally related, the same variables may have similar effects on their activity. But this is not always the case as under different conditions their activity levels may not match (Rungruangsak-Torrissen et al., 2006). For instance, in studies with different feed type, hormonal and light treatments, as well as starvation/refeeding trials, there were different relationships between the two enzymes (Le Moullac et al., 1996; Lemieux et al. 1999; El-Saidy et al., 2000; Sunde et al., 2001; Rungruangsak-Torrissen et al., 2006). Furthermore, production of TRY is never terminated even through fasting (Lehnert and Johnson, 2002) and often the link between the two enzymes can be clearly detected during those periods while the strength of the relationship weakens and may disappear when feeding recommences and GR increases (Rungruangsak Torrissen and Male, 2000; Rungruangsak-Torrissen et al., 2006).

In the present study both species showed positive correlation between TRY and CHY, independent of feeding levels in snapper (relationship was preserved even in summer when feeding was most

intense), and in YEM (feed intake was recorded throughout the entire study). In addition, the pattern with regards to the relationship with GR suggesting that TRY activity as well as TRY/CHY ratio increase parallel with GR has not been observed in the present study.

Moreover, in the test species both levels of serine proteases were low compared with other studies and they were at similar levels, so that the TRY/CHY ratios were likewise relatively low (i.e. ~1.1 and ~0.8 for snapper and YEM respectively). Atlantic mackerel (*Scomber scombrus*) is a schooling pelagic fish with a rapid growth profile that during steady growth exhibits low values of TRY/CHY ratio. Besides, they have pronounced visceral fat deposition rates (Rungruangsak-Torrissen and Fosseidengen, 2007). This description resembles YEM in the present study whose growth extended without ceasing throughout the feeding experiment, and TRY/CHY ratio was generally low (kept below 1.0), meaning that CHY activity was almost entirely above TRY. Therefore, it appears, at least for these two species (mackerel and YEM) and possibly for some other pelagic schooling fish, that importance of CHY for digestion and growth is equal to or exceeds that of TRY, while for snapper TRY and CHY may be similarly important for digestion and growth irrespective of levels of feed intake and growth performance.

5.4.3 Seasonal effects on test species enzyme activities

5.4.3.1 Aerobic capacity and seasonal dynamics

Temperature as the most influential seasonal factor affects mitochondrial physiology in a number of cold active eurythermal teleosts in such a way that aerobic capacity in muscle tissues is increased as a response to cold acclimation (Guderley and St-Pierre, 2002; Hochachka and Somero, 2002; Guderley, 2004). Tissue aerobic capacity is adjusted to seasonal temperature fluctuations by altering mitochondrial capacities, changing mitochondrial volume density or a combination of the two (St-Pierre et al., 1998).

In the present study seasonal temperature had varying effects on the aerobic capacity of the test species, which was generally stronger in snapper than YEM. In snapper there were no apparent signs of winter acclimation since no major seasonal adjustments (no thermal compensation but rather thermal conforming) were observed in WM, suggesting that there were no changes in mitochondrial volume density, nor major changes in mitochondrial capacities. However, the activity of CCO went down earlier in summer and likewise went up earlier in late winter relative to CS and the temperature curve, so some minor mitochondrial modifications did occur. An earlier seasonal adjustment in CCO activity may imply that snapper sensory systems detected a seasonal thermal and photo signal which

stimulated change in mitochondria to be aerobically/metabolically ready for the forthcoming season (van Dijk et al., 2005). But in RM, snapper appear to weakly compensate when in cold, possibly by increasing mitochondrial density volume which was assumed based on a positive correlation between CCO and CS in RM. However, since the relationship was not strong some other compensatory means may be involved, possibly alteration in phospholipid membrane as the most common thermal acclimation adjustment strategy (Guderley, 2004).

In general, there is a scarcity of primary literature describing seasonal effects on metabolic activity of the test species. Nevertheless, Majed et al., (2002b) provides some baseline information regarding annual cycling of LDH and CS in wild snapper. They found that only CS demonstrated seasonal effects due to positive correlation with annual temperature oscillations, also resembling annual growth pattern, which strictly coincides with the CS activity observed for WM snapper in the present study. They argued that a possible reason for the pattern where CS activity diminishes in winter, as was proposed for barred sand bass *Paralabrax nebulifer* exposed to starvation (Lowery and Somero, 1990), was because of a reduction in protein synthesis as an expression of a strategy for energy preservation during the cold season when feed intake and GR are minimised. This explanation may be also applicable for the present study snapper.

In terms of β-oxidation represented by the activity of HOAD, clear seasonal effects were found in RM of Atlantic cod (Pelletier et al., 1993) and rainbow trout (St-Pierre et al., 1998; Egginton, et al., 2000) indicating an increased importance of lipid catabolism during winter/cold period (Thibaoult et al., 1997; Guderley, 2004). The present data for both test species were inconsistent with these findings since the thermal effect was not present and changes observed were likely not due to cold acclimation. Nevertheless, RM HOAD in snapper showed seasonal effects, which coincided with the aforesaid relationship between enzymes activities and snapper condition where late autumn was documented as a period with highest condition as well as enzymes activity. And in the same note annual YEM HOAD activity in RM reflected dynamics of energy reserves.

When snapper aerobic activities in musculature were compared with those of phylogenetically related *S. aurata*, some similarities as well as major differences were found. Firstly, *S. aurata* belong to a group of temperate fish that do not exhibit processes that reflect a conservation of aerobic capacity in muscle tissues due to acclimation to cold, apart from HOAD activity in RM (Kyprianou et al., 2010). This was generally true for snapper; however, an exception was a response of RM where partial temperature compensation of CCO and CS activities was evident. Snapper HOAD activity in RM was affected with season/temperature but not in a way as in *S. aurata* where it was obvious that oxidation of free fatty acids, to support winter ATP turnover, was emphasised (Kyprianou et al., 2010). Snapper HOAD peaked in late autumn and the activity dropped through winter, similarly as in WM, indicating minor importance of fat oxidation in snapper musculature as an energy generation pathway

in winter contrary to the response commonly observed in temperate species (Guderley, 2004). Therefore, the general similarity between *S. aurata* and snapper is that both predominantly thermally conform since a depression in metabolic activity as a response to a temperature decrease was evident (Kyprianou et al., 2010; Requena et al., 1997; this study).

5.4.3.2 Anaerobic (glycolytic) capacity and seasonal dynamics

Glycolytic enzymes have also shown sensitivity to seasonal temperature change, such as in Atlantic cod (Pelletier et al., 1993a) and rainbow trout (Thibault et al., 1997). In trout glycolytic metabolism, phosphofructokinase and LDH had higher activity in the warm than the cold season in both white and red muscle (Thibault et al., 1997). In snapper the activity of LDH in WM and RM and PK in RM showed the highest activity in autumn corresponding with the highest annual snapper condition, while PK in WM had a general tendency to increase without demonstrating seasonal effects. The pattern when glycolytic activity was not at its highest in synchrony with the highest annual temperatures contrasts with *S. aurata* where increased activity of LDH and PK were associated with temperatures above 20 – 22°C as a part of a thermal stress response when increased dependence on anaerobic glycolysis was emphasised (Feidantsis et al., 2009). Likewise, snapper glycolytic dynamics in muscles did not show any resemblance to *S. aurata* when experiencing lowest winter temperatures where, again, anaerobic glycolytic activity was boosted below 14°C (Kyprianou et al., 2010).

Activities of both LDH and PK in YEM WM strongly correlated with visceral fat index. However, glycolytic activity in YEM RM seemed to be affected with annual temperature fluctuation since the appearance of thermal compensation without experiencing positive correlation with VLI was obvious. This may place YEM amongst few fish species where seasonal compensatory effects were also reported for glycolytic enzymes, as was the case for lake whitefish (*Coregonus clupeaforrnis*) (Blier and Guderley, 1988). This positive thermal compensation may be important for maintenance of locomotory capacity at low temperatures (Blier and Guderley, 1988). Furthermore, the glycolytic dynamics in YEM RM may suggest that RM, in contrast to WM, is likely not a site for cytosolic depositing of proteins as stores, which cannot be said for mitochondrial matrix proteins such as oxidative enzymes (CS and HOAD) since they also positively correlated with VLI.

5.4.3.3 Digestive capacity and seasonal dynamics

The annual dynamics of digestive enzymes demonstrated a distinct but markedly different seasonal pattern in the test species. The pattern that has been noted, for example, for Atlantic salmon

(Einarsson et al., 1997) where secretion of TRY was the lowest in winter and highest in summer was only partially followed in snapper while, in YEM, both TRY and CHY activities were highest in winter and lowest in summer. Negative correlation with annual temperature fluctuation as found in YEM, indicates that the highest requirement for proteolytic activity was in winter supposedly to offset the negative impact of low temperatures on growth capacity where enhanced activities of digestive enzymes ensured that the nutrient supply was sufficient to sustain continuous, overwintering YEM growth. In contrast, snapper digestive enzyme activity demonstrated a peculiar seasonal dynamic with distinct peaks in spring and autumn corresponding with resumption of growth after winter break and a period of intense energy stores deposition before winter respectively.

Serine proteases play a crucial part in nutrient influx and were proposed as regulators of growth via their control of amino acid supply (Torrissen et al., 1994). Their expression is affected by a combination of many internal (growth hormone – Lemieux et al., 1999; cholecystokinin – Einarsson and Davies, 1996; gene manipulation - Sunde et al. 2001; Blier et al. 2002) and external factors (light regime – Sunde et al., 2001); feeding regime, dietary properties and temperature (Rungruangsak Torrissen and Male, 2000; Sunde et al., 2004). Since a combination of factors are involved in regulation of their functioning, it is expected rather than to find a universal pattern in response to seasonal environmental change, there would be changes that would best support growth potential and ecological lifestyle of a particular species, as has been observed in the present study.

5.4.4 Enzyme activity ratios and metabolic differences between test species

5.4.4.1 Aerobic ratios – CS/CCO – importance of the Krebs cycle relative to ETC

When overall annual averages were concerned, the CS/CCO ratio in YEM musculature was higher in RM than in white, as was also observed for rainbow trout (Guderley and Gawlicka, 1992). However, in snapper the ratio was inversed – higher in WM relative to RM, while the ratio in intestines, for example, was identical between the test species. In addition, the ratio was larger in YEM, especially in RM (over four times). These results indicate that YEM muscle tissues overall rely considerably more on the Krebs cycle when compared with ETC, and since the capacity of ETC (as represented by CCO activity) has been recurrently found to significantly exceed demand for ATP production in both WM (Blier et al., 1997) and RM (Blier and Lemiex, 2001), and therefore CCO activity cannot be considered to set limitation over mitochondrial respiration, it can be suggested that YEM had overall greater demand for aerobic energy turnover in musculature than snapper, which is met by augmented activity of the Krebs cycle, and this seems to be particularly important in RM.

When CS/CCO ratio was monitored monthly throughout the study in snapper WM it strongly correlated with the fish condition, VLI and glycogen stores, while in RM the ratio appeared unchanged. In contrast, no particular pattern was observed for CS/CCO in YEM WM; however, in RM, similar to snapper WM, and as it has been repeatedly reported throughout the discussion, the ratio demonstrated strong association with the condition of energy stores. In the case of rainbow trout kept at maintenance feeding regime the ratio in WM was distinctly affected by temperature, being higher in cold than warm, but in agreement with snapper no change was observed in RM (Guderley and Gawlicka, 1992). The similarity between visual predators such as snapper (Robinson et al., 2011) and rainbow trout (Ware, 1972) was obvious in RM, but inconsistency in WM apart from phylogenetic and behavioural factors may also stem from experimental conditions such as differences in dietary regime. This could be suggested on the basis that both test species were reared in identical conditions with unlimited food supply and despite major differences between the two in biological and ecological traits they both put emphasis on their condition and energy reserves which were reflected in activity of major metabolic pathways in their musculature. In addition, Guderley (1990) argued that thermal compensation may be masked by reproductive (relevant for YEM as later in the study many fish demonstrated advanced signs of gonadal development) and feeding activities (relevant to both snapper and YEM). Moreover, Guderley (1990) also suggested that it is required at least 10°C below the optimal temperature for sustained swimming for thermal compensation to be required to support locomotory activity in the cold. Since optimum temperature for sustained swimming in snapper was estimated around 17°C but above 22°C for YEM (chapter 3) and the lowest temperature experienced during the winter did not go below 8°C, it is plausible that snapper did not required to compensate; however thermal compensation may be important for YEM if Guderley (1990) suggestion can be extrapolated to the present study species.

5.4.4.2 Aerobic ratios: HOAD/CS and HOAD/CCO – relative importance of β -oxidation to aerobic metabolism and ETC

Annual average of both HOAD/CS and HOAD/CCO ratios demonstrated for both test species greater importance of β -oxidation/lipid catabolism in RM than in WM, which was expected as this is the pattern commonly observed in fish musculature (Guderley and Gawlicka, 1992; Cordiner and Egginton, 1997; Guderley, 2004). However, YEM had distinctly higher HOAD/CCO ratio in both muscle types and particularly in RM where, similar to CS/CCO, the ratio was about four times larger than in snapper, suggesting much greater importance of lipid catabolism for aerobic ATP production in YEM musculature compared to snapper. Although higher HOAD activity was observed for both WM and RM, the interspecific differences were based mainly on much lower CCO activity in both YEM muscle types, particularly in RM, compared with corresponding snapper tissues. In addition, since both

mitochondrial matrix enzymes (HOAD and CS) due to enhanced demand for aerobic energy production in RM distinctly increase relative to CCO activity; this may be another piece of evidence, at least for YEM RM, that the capacity of ETC to supply ATP is above the requirement for highly energy demanding tasks such as myosin contraction or protein synthesis (Blier and Lemiex, 2001).

Besides, in both YEM muscle tissues HOAD/CCO, and in RM HOAD/CS, observed monthly over a year, demonstrated thermal compensation in YEM aerobic capacity, which was not evident when enzymes were examined individually, suggesting a specific strategy of YEM to respond to cold and adjust its oxidative capacities to maintain the energy production required for both replenishment of energy stores and perpetual growth.

5.4.4.3 Aerobic/anaerobic ratios: LDH/CS – measure of relative anaerobic vs. aerobic capacity

For both test species LDH/CS ratio emphasised, based on large differences between values in two muscle types, much greater importance of anaerobic over aerobic metabolism in WM than in RM. When annual averages were concerned, in both muscle types a slight, still significantly higher reliance on anaerobic metabolism was observed for snapper. However, the ratio's annual dynamic (measured monthly) revealed more specific differences between the two species. In the relative of snapper, *S. aurata,* the LDH/CS ratio was seasonally affected in a way that during winter the ratio increased in oxidative tissues (RM and heart) indicating greater importance of anaerobic metabolism as a strategy for coping with stressful cold conditions (Kyprianou et al., 2010). However, snapper data could not suggest such a strategy, neither based on annual LDH activity alone or as LDH/CS ratio, despite snapper experiencing much lower winter temperatures than 12–14 °C as a metabolic threshold proposed for both species (i.e. *S. aurata*, Ibarz et al., 2003, 2007; Kyprianou et al., 2010 and snapper, chapter 3, this study). Instead LDH/CS in both muscle types were the highest in late summer/autumn corresponding with the annual peak in snapper condition. But in YEM RM, the ratio was the lowest in summer and highest in winter when YEM possibly utilised the same strategy as *S. aurata* when faced with low winter temperatures. On the other hand, in YEM WM, the LDH/CS ratio linearly decreased as VLI increased suggesting that the importance of aerobic metabolism was enhanced with increase in energy reserves.

5.4.4.4 Aerobic/anaerobic glycolysis – PK/LDH ratio

In terms of relative importance of aerobic over anaerobic glycolysis, the ratio of PK/LDH was calculated due to PK's involvement in both pathways. The ratio is particularly informative for

interspecific comparisons if LDH/CS ratios are at similar levels (Hochachka et al., 1982), which in the current study was the case for both test species. Based on annual averages YEM demonstrated greater importance of aerobic glycolysis in both muscles than snapper; however, not significantly in WM. In terms of seasonal effects, in rainbow trout Thibaoult et al. (1997) found that aerobic glycolysis in WM was enhanced in the summer period. That could not be supported in snapper WM where somewhat opposite trend occurred, but it was the case for RM since the ratio correlated with annual temperature oscillation. Overall, this indicates that glycolytic capacities of snapper musculature undergo seasonal reorganization. However, in YEM, again, the annual trajectory of PK/LDH ratio was modified in support of overall body energy reserves.

5.4.5 Annual metabolites dynamics

5.4.5.1 Uncommon levels of tissue metabolites

Some levels of tissue metabolites appeared to be substantially above or below those reported in previous studies involving snapper and YEM (Black, 2002; Black et al., 2004; Tuckey et al., 2012; Cook and Herbert, 2012). In particular, lactate and glucose concentration in all three examined tissues in snapper and in YEM appeared higher than expected and glycogen stores in snapper WM were below commonly published, with an exception of Coxon (2014) who also reported both high lactate and low glycogen levels for snapper. The sampling procedure practiced in the present work was, however, comparable with Black et al. (2004) and Tuckey et al. (2012) where fish, however substantially larger, were exposed to the same type of anaesthetic (AQUI-S™) for the similar period as in the present study; and the euthanasia technique by brain pithing with an *iki jime* instrument was the same across the studies. The only notable difference was in the amount of time (i.e. ~20 minutes specimen^{-1} times 7–8 fish sampling day^{-1}) required to obtain the morphometric measurements and the array of tissue samples needed from an individual fish biochemical profiling. Nevertheless, the sampling duration following euthanasia had no effects on either fish WM lactate levels, weak or no effect on RM lactate, and only stronger effects were observed for liver lactate where similarly for both species lactate levels increased by ~45%. In addition, glycogen stores were not affected by the sampling/dissection time in any of the three snapper tissues, but in the case of YEM WM and RM glycogen levels dropped significantly by ~25%, but only in the first 3 months, when the highest concentrations of glycogen across the entire study were observed. From this it becomes obvious that distinctly high levels of lactate, particularly in snapper and YEM WM, as well as low levels of glycogen in snapper WM are not likely an artefact of the prolonged sampling. In addition, it is also unlikely that fish in the experiment were exposed to chronic stress, which can affect tissue metabolism and associated levels of related metabolites, in which case the tissue changes would be accompanied with a reduced feed intake and

impaired growth performance (Gregory and Wood, 1999). However, the feeding behaviour and the growth were unfolding as expected (i.e. both species have surpassed growth rates of equivalent wild fish, see chapter 6). High WM lactate in rested snapper was also reported in some earlier work (see Lowe et al., 1993) and it is not uncommon in some other species such as silver catfish (*Rhamdia quelen*, 60–70 μmol of lactate g^{-1}; Lermen et al 2004). In addition, Coxon (2014) suggested, since no particular cause for the atypical metabolic profile was found, that the pattern may have stemmed from inherently high levels of lactate and low levels of glycogen in the snapper used. Tuckey et al. (2012) also reported high WM lactate and since Coxon (2014), Tuckey et al. (2012) and the present study utilised snapper from the same source, her genetically related insight may be possible. However, that cannot explain high lactate levels in YEM WM.

There is another explanation that can encompass the apparent lactate/glycogen anomaly of both test species. Previously, it has been identified that activities of many enzymes in this study increased parallel to fish condition and building up of energy stores. That was especially true for LDH, since it has been recognised as a measure of muscle protein storage and energy status (Houlihan et al., 1988; Gauthier et al., 2008) and its activity demonstrated particularly strong correlation with condition (snapper) and VLI (YEM) in WM. Besides, LDH activity in snapper WM and RM, and YEM WM also correlated with lactate content in the tissue. Overall, levels of LDH were exceptionally high (i.e. ~450 μmol g^{-1} in Majed et al., (2002b) snapper vs. ~1000 in the current study; for YEM there is no published WM lactate concentration, nevertheless in this study it was ~1300 μmol g^{-1}) likely reflecting unrestricted feeding experimental conditions. Therefore, maximum levels of feed intake may be responsible for high levels of LDH that turned a greater proportion of pyruvate into levels of lactate observed in this study. Besides, glycogen correlated (negatively) with both LDH and lactate in YEM WM; however, these relationships were weak or not existent in snapper WM.

In addition, there are many aspects of experimental procedure/design that can cause differences in tissue metabolic profiles observed amongst studies, such as effects of use vs. not use of anaesthesia (Iwama et al., 1989; Schwalme and Mackay, 1990); effects of fish size and ontogeny (Goolish, 1991; Kiessling et al., 1991); effects of rearing conditions and stocking density (Sangiao-Alvarellos et al., 2005; Roncarati et al., 2006; Blasco et al., 2015); effects of animal origin – hatchery reared vs. wild (McDonald et al., 1998); and effects of diet type and ration size (Castro et al., 2016) to name a few. Therefore, it is plausible that the levels of tissue metabolites observed in the current study (apart from those affected by sampling duration) were actual values, thus the metabolite concentrations measured were acquired prior to sampling as a reflection of specific conditions experimental fish were exposed throughout the course of the study.

5.4.5.2 Lactate annual dynamics

Compared with mammals, fish appear to have a relatively poor ability to use carbohydrate and generally low metabolic rates (Lermen et al., 2004). Nevertheless, a unique feature of fish is a comparably high capacity for anaerobic metabolism, particularly in WM, tissue characterised by low levels of perfusion. WM makes a greater proportion of skeletal musculature, and all together they contribute up to 60% of fish body mass (Bennett, 1978). Therefore, lactate production can be relatively high in fish and it is believed to be an important intermediate metabolite finding its purpose as an oxidative fuel, gluconeogenic substrate and signalling molecule (Gladden, 2004; Philp et al., 2005; Omlin et al., 2014).

Lactate dynamics in all three examined tissues in the test species varied in their annual trajectories though only in snapper RM and liver a clear seasonal pattern was observed. A connection between liver and RM lactate in snapper was likely established via circulation, while the amount of lactate in RM was probably a reflection of levels of fish activity – being the highest in summer and lowest in winter. This pattern of seasonal change in activity has been often reported for temperate fish (Rahel et al., 1996; Hammerson, 2004; Brown et al., 2004). However, in WM, lactate concentration reflected the annual trajectory of LDH activity, demonstrating a possible link between snapper condition and energy stores.

Black (2002) reported no difference in lactate content between summer and winter YEM WM. The WM lactate in the current study was the lowest in autumn and highest in early summer. But, since the overall annual lactate trajectory correlated with LDH and VLI, the dynamics of YEM WM lactate may therefore be an artefact of a metabolic propensity related to energy deposition and not the product of seasonal change. However, lactate in YEM RM, as mentioned earlier, may reflect increased reliance on anaerobic glycolysis in the cold (Kyprianou et al., 2010), while for liver lactate no particular seasonal pattern was observed but rather its content was positively associated with GR. Higher levels of activity (i.e. feeding) may be related to higher GR (Houlihan et al., 2001). This may result in higher levels of tissue lactate, which in turn may be reflected in plasma lactate levels and the rate of its uptake by liver. As lactate is a highly valuable gluconeogenic substrate for fish liver (Hemre et al., 2002) an equivalent rise in glucose may be expected. Any glucose in excess of the current requirements may be rapidly/instantly (since there were no correlation between liver lactate and glucose) turned into lipids (Polacof et al., 2012) rather than glycogen and used systematically for the liver work, as lipid seems to be a preferable form of energy stores for YEM in this study. In support of this notion strong positive correlation was observed between liver lactate and HOAD confirming the link with the lipid metabolism.

5.4.5.3 Glucose and glycogen annual dynamics

In this study season clearly affected snapper annual glucose/glycogen dynamics, while this could not be suggested for YEM. In YEM, however, strong intra-tissue association between glucose and glycogen was pronounced, since in all three tested tissues positive correlation was observed. In snapper, glucose concentration in RM and liver was negatively correlated with sea-water temperature, being the lowest in summer and highest in winter, indicating a possible greater need for glucose, as an energy source, during the cold period in the respective tissues. On the other hand, in WM, glucose concentration peaked in autumn, which was in synchrony with the pre-wintering replenishment of energy stores, as it positively correlated with snapper condition, VLI and glycogen. In addition, annual snapper glycogen dynamics followed fish condition in all three selected tissues, exhibiting the same pattern with the highest levels observed just before winter.

Many temperate species, including snapper, markedly reduce feed intake and consequently growth rates through the coldest months (Pottinger et al., 2003; Ibarz et al., 2007). During that time the accumulated energy stores play an essential role for maintaining homeostasis. However, not only glycogen in liver but also in musculature was massively mobilised through that period and this cannot be attributed to enhanced muscular activity as decreased feed intake/fasting is associated with decrease in locomotory activity (Johnston, 1981; van Dijk et al., 2002). In addition, many fish species first utilise lipid reserves in relation to food deprivation, irrespective of the preferable site for their deposition – liver, abdominal cavity or muscle (Black and Love, 1986; Sargent et al., 1989; van Dijk et al., 2005). This principle is not consistent with the snapper data, since both major energy stores – visceral fat and tissue glycogen simultaneously depleted throughout the winter as demonstrated with the strong positive correlation amongst the variables. The same seasonal pattern in liver glycogen was also reported for *S. aurata* (Vargas-Chacoff et al., 2009a).

Rather unique glycogen dynamics have been observed in YEM. Muscle growth in fish after a reinstitution of feeding may be different to the growth when food supply is not disrupted, since the catch-up growth may be first characterised with synthesis of tissue with lower energy content (i.e. glycogen has 17 kJ /g compared to 39 kJ/g for fat, Brett and Groves, 1979) (van Dijk et al., 2005). In support of this notion it is often found that glycogen stores are reinstated relatively quickly after starvation, demonstrating a strategy for rapid and efficient energy restoration that can be subsequently utilised for production of different tissues and their components (van Dijk et al., 2005). Mendez and Wieser, (1993) found in juvenile roach (*Rutilus rutilus*) six to nine times higher glycogen content after a week of refeeding following several weeks of starvation. A comparably high glycogen content has been also observed in a few other species (e.g. cod *Gadus morhua*, Black and Love, 1986;

pike *Esox lucius*, Ince and Thorpe, 1976). Similarly, YEM had relatively high glycogen levels within the first 2 months of the growth trials in both RM and WM, which plausibly can be attributed to the aforementioned principle. YEM for the purposes of this study were obtained from the wild and kept in PFR facilities for approximately a month under a maintenance feeding regime and subsequently they entered the experimental procedure characterised with unlimited food supply. This transition from wild to *ad libitum* food sources may trigger a response in replenishing glycogen stores to exceed more than two times levels that have been observed for YEM kept on maintenance feeding regime (i.e. maintenance feeding – ~35 μmol g^{-1} glucosyl units in WM in Black, 2002; ~80 and 240 μmol g^{-1} glucosyl units in WM and RM respectively at the beginning of the feeding trials). However, after high initial levels, glycogen dropped rapidly within the first 3 months in the study negatively correlating with VLI, and it had likely been replaced with lipids (van Dijk et al., 2005) in all 3 tested tissues. This made YEM to operate at very low levels of glycogen for the greater part of the study; however, the glucose levels in corresponding tissues were comparably high (c.f. Pujante et al., 2015), indicating that reliance on glucose metabolism was still evident. This may suggest that in an event of food shortage or prolonged stress (e.g. hypoxia) YEM would have possibly experienced adverse lag time effects since the majority of stores were likely in the form of lipids from which extraction of energy is much slower and costlier compared to glycogen (Mommsen, 2001). Nevertheless, the scenario observed in the present study may be unique to the food unlimited conditions, since, for instance, in thick-lipped grey mullet (*Chelon labrosus*) kept at maintenance feeding regime (i.e. 1% body mass with commercial pellets a day) liver glycogen was at similar level as in YEM at the very start of the experiment (~280 μmol g^{-1}) (Pujante et al., 2015). From that level, glycogen in liver of *C. labrosus* was rapidly mobilised during starvation for the maintenance of metabolic functions, as a typical response also observed in many other species (e.g. rainbow trout – Figueroa et al., 2000; *Dicentrarchus labrax* – Pérez-Jiménez et al., 2007; *S. aurata* – Vargas-Chacoff et al., 2009b), indicating its importance as a readily available energy source also for mugilids.

5.4.6 Concluding remarks

Summary of the main findings:

- **Muscle aerobic activity** - WM activity in YEM was likely a reflection of augmented energy deposition due to strong positive correlation between enzymatic activity (especially CS) and VLI. This may be a response to favourable food-unlimited rearing conditions that possibly allowed YEM to fill up all energy stores. However, CS activity was positively associated with GR in snapper WM likely showing its direct involvement in growth. Seasonal temperature differently affected the aerobic capacity of the test species and effects were generally stronger in snapper than in YEM. Nevertheless,

no apparent signs of winter acclimation were obvious for snapper WM since no major seasonal readjustments in terms of temperature compensation were observed; however, this was true for RM.

- **Intestinal aerobic activity** – the data for both species demonstrated a lack of evidence that aerobic capacity of intestines was limiting to GR, contrary what has been reported for other teleosts (for examples see text above).

- **Muscle glycolytic enzymes** – In general, snapper glycolytic activity (especially LDH in WM and RM and PK in RM) closely followed fish condition and VLI. Thus, the level of glycolytic activity in snapper muscle tissues (mostly in WM) is predominantly an expression of energy status and reserves. In YEM, both glycolytic enzymes demonstrated, as seen for oxidative enzymes, a pattern of negative correlation with GR, which again, due to a much stronger relationship of glycolytic enzymes with VLI is likely, an integral part of overall body energy reserves dynamics. However, glycolytic activity in YEM RM seemed to be affected by annual temperature.

- **Seasonal effects on muscle lipid catabolism** – The present data for both test species were inconsistent with findings from other teleosts of increased importance of lipid catabolism during winter, since the thermal effect was not present, and changes observed were likely not due to cold acclimation.

- **Seasonal effects on digestive capacity** – In snapper this was rather complex without an obvious pattern, while in YEM both TRY and CHY activities were highest in winter and lowest in summer, likely indicating greater requirement for proteolytic activity in coldest months supposedly to offset the negative impact of low temperatures on growth performance.

- **CS/CCO** – this ratio data suggests that YEM had overall greater demand for aerobic energy turnover in muscle tissues than snapper. This is demonstrated by enhanced activity of the Krebs cycle (i.e. CS activity), which was particularly emphasised in RM.

- **HOAD/CCO** – YEM had this ratio greater in both muscle types, yet considerabley higher in RM, suggesting much greater importance of lipid catabolism for energy production in YEM musculature compared with snapper.

- **LDH/CS** – in both muscle types, this ratio demonstrated overall a slight, still significantly greater dependence on anaerobic metabolism in snapper than in YEM.

- **PK/LDH** – contrary to LDH/CS, the PK/LFH ratio demonstrated generally greater importance of aerobic glycolysis in both YEM muscles relative to snapper.

- **Lactate** – dynamics in all three tissues in both test species varied in their annual trajectories. However, a positive correlation with temperature was seen only in snapper RM and liver. In YEM, the overall annual lactate trajectory, correlated with LDH and VLI, as likely an artefact of a metabolic propensity related to energy deposition.

- **Glucose/glycogen** – season evidently affected snapper annual glucose/glycogen dynamics, being aligned with pre-wintering conditioning; however, not so in YEM. In YEM the unusual annual glucose/glycogen trajectory was likely coupled with the preference for lipid and protein as energy storage and this may be unique to the food unlimited conditions.

In the case of snapper clear seasonal effects were observed. They were more in terms of conforming (e.g. CCO WM, CS WM/intestine, lactate RM/liver) and thus following the annual temperature curve and less in compensating for temperature effects (e.g. CCO/CS RM). However, in many instances metabolic focus pointed towards the preparation for winter by maximising condition and energy reserves prior to onset of the adverse cold period, as snapper and S. aurata (Ibarz et al., 2003; 2007), and possibly other sparids are distinctly susceptible to low temperatures.

In YEM, with a few exceptions, generally there was only weak evidence to suggest that season per se had any major effects on their biochemical profile. Similar to snapper, but not in association with pre-wintering preparation, the focus also seemed to be centred around maximising energy stores and the build-up was likely simultaneous across all preferable energy store types, which for YEM appeared to be lipids and proteins.

The main driver of YEM biochemical, as well as growth profile was likely the unrestricted feeding regime that experimental animals were exposed to. YEM evidently possess a capacity to reorganise thier metabolic machinery to maximise benefites when feeding conditions improve as food availability may be the most challenging factor that they may face in the wild. This can be also applicable to snapper; however, the influence of seasonal temperature oscillation may be equally important affecting snapper performance in synergy with levels of food supply.

CHAPTER 6

Food limitation in wild versus maximum growth rates of snapper and YEM

6.1 Introduction

6.1.1 Fish mass and length data in commercial stock assessment and their utilisation

The simplest and quickest form of assessing the state of certain fish stocks either in the field (i.e. on fishing vessels) or in aquaculture facilities is by taking length and mass measurements for subsequent analysis and projections. This has been considered a backbone procedure in any fishery research, stock monitoring programmes and management procedures (Anderson and Neumann, 1996; Blackwell et al., 2000). There are two main objectives as to how the data can be utilised. First, knowing the relationship between the two variables, the arithmetic approach allows the prediction of one variable based on the other. Therefore, estimates of biomass can be quickly and accurately made for the species for which stocks mass-length relationship (MLR) is known if only length data is obtained at the time. And second, by using the mass-length data to express fitness or condition level as an indicator or proxy for wellbeing and gonad stage representing reproductive condition of a population (Tesch, 1968; Blackwell et al., 2000). These two principles have been exploited in fishery sciences for over a century and even though the usefulness of the relationship has been challenged lately in some scientific circles (e.g. see Hilborn and Walters, 2001), the mass and length data and their relationship are still considered important information and is required as a base for understanding ecological and stock productivity traits. However, correct use of the data is essential (Froese, 2006; Ogle and Windfield, 2009).

6.1.1.1 Mass – length relationship (MLR)

The era of integrating information about mass and length in studying fish biology and growth started in the early 20th century with Thomas Wemyss Fulton who was first to implement the cube

law that states that volume increases as the cube of linear dimensions, or in practical terms, when a fish doubles in length it increases its mass eight times (Froese, 2006). Despite realising that fish often do not follow the rules of the law, Fulton was an advocate of its implementation to all fish (Froese, 2006; Nash et al., 2006). His determination eventually resulted in the well-known Fulton's condition factor (*K*, for equation see 2.2.4.1.1) to become the most widely used condition index in fishery science/industry (Blackwell et al., 2000; Froese, 2006). The Fulton's *K*, in agreement with the cube law, implies isometric growth which is designated with a fixed mass–length ratio (i.e. exponent *b* = 3.0) where it is assumed that fish do not change shape and form as they grow. That means that the form factor effect is neglected, and more elongated species appear to have lower condition than short and deep shape fish, which would be erroneously suggested by *K* (Blackwell et al., 2000). Not long after postulation of *K*, several researchers identified that the better explanation of MLR was by estimating the length exponent *b* as a second parameter (first is intercept *a*) in the relationship rather than using a fixed value of 3.0, which in turn gave birth to the MLR as we know it in its arithmetic and logarithmically transformed form (for details see 2.2.4.1.1).

MLR is particularly useful in modelling aquatic ecosystems, especially where only frequency distribution and length data for certain species are known as a platform for biomass estimation (Kulbicki et al., 2005). Even though the number of species with known MLR seems to be limited, thus slowing down some ecosystem modelling predictions (Kulbicki et al., 2005), Froese (2006) conducted an extensive *meta*-analysis and managed to encompass about 4000 studies describing MLR of nearly 2000 fish species. Since then many more have been added to the data base. His key findings were that the overall inclination of most fish was to exhibit minor positive allometric growth (i.e. growth is displayed as an increase in relative body thickness or plumpness) since median *b* value of 3.03 was found to be significantly greater than isometric value of 3.0. More than 80% of all species demonstrated some sort of allometric growth (positive or negative) and the majority of examined exponent *b* values were placed between the expected 2.5 and 3.5 envelope (Froese, 2006). In addition, MLR has been utilised as an aid in comparing fish growth and morphological traits among areas, and as a complement to reproduction and feeding studies (Petrakis and Stergiou 1995; Moutopoulos and Stergiou, 2002; Froese, 2006).

6.1.2 Scales, source for fish aging and back-calculation of past length at age

Estimating the age of living organisms is an important subject vital to many fields of science, conservation and stock management (Hoxmeier et al., 2001; Jobling, 2008). Annual or daily growth marks in various animal structures (e.g. coral skeletons, Dodge and Thomson, 1974; bivalve shells, Dodge and Thomson, 1974; cricket exoskeletons, Zuk, 1987; tortoise scutes, Germano, 1998;

mammalian teeth, Goren et al., 1987) that produce periodic growth increments have been regularly used for age and past growth rate estimation (Campana, 2001). In relation to fish, several calcified structures have been utilised for aging purposes with various degree of precision. The most common are vertebrae, opercula (a hard, plate-like, bony flap that covers the gills of a bony fish), cleithra (a bone that extends from the base of the pectoral fin forming the posterior edge of the gill chamber), fin rays, scales and otoliths (fish ear bones) (Campana, 2001). Moreover, fishery related annual ageing requirements can be obtained by utilisation of any calcified/bony structure in the fish; however, the otoliths and scales have been shown to be the most accurate and therefore the most frequently used (Casselman, 1987; DeVries and Frie, 1996). Age determination via otoliths is considered to be generally advantageous over scales, especially when adult fish are concerned, since calcium deposition in otoliths has been found to be of higher importance than in scales (Carlander, 1987), and otoliths continue to grow as fish age (Beamish and McFarlane, 1983; Casselman, 1987). Nevertheless, ageing by scales is a well-established practice in temperate (higher latitude) fish and in many instances a preferable method since their collection is nonlethal and it is easily carried out in the field (Chilton and Stocker, 1987; Kruse et al., 1993). Scale preparation and analytical processing is likewise relatively simple compared to other methods (Hoxmeier et al., 2001).

Age and past length determination is based on identification and appropriate measurements of several key scale features namely focus, radii, and annuli (Fig. 6.1). The focus is the starting point for determination of all required measurements (i.e. the scale radius and annulus increment radii) since it is considered to be an initial primordial structure around which a scale developed through its lifetime (Zale et al., 2012). The size of the scale radius (the scale radii are grooves that extend from the focus to the edge of the scale) is required for establishing the relationship with body length, which in turn will convey the information about what approach (i.e. Dahl-Lea or Fraser-Lee, for details see the method section 6.2.2) is appropriate for estimating past length at age of the fish of interest (Zale et al., 2012).

Annuli, the key for unlocking growth in the past, are the ring like structures that form once a year, thus representing the turning point of growth from one year to the next. The circular ripple lines – circuli (Fig. 6.1), as scale growing increments, may not be laid down in the same fashion (i.e. observed as the distance between circuli) all year around, but they rather correspond with the growth in length (Jobling, 2008). Therefore, for temperate fish during winter when growth is generally reduced, circuli are more crowded and when feeding is reinstated in spring the spacing between circuli become greater (Devries and Frie, 1996; Campana, 2001). This distinct ring like feature on a scale depicting transition between slow and rapid growth represents an annulus (Zale et al., 2012; Fig. 6.1). The inner edge of an annulus represents the beginning of spring, and the spacing between two annuli stands for the growth of one calendar year and it is proportional to the annual length increase of a given fish (Jobling, 2008; Zale et al., 2012).

In the past, several issues have been described regarding the use of scales for ageing and calculating past length, such as false annuli, difficulty with identifying the first annulus, crowding of annuli at the outer edge in older (matured) fish especially in slow-growing species (Welch et al. 1993; Hoxmeier et al., 2001), often causing age underestimation in older fish (Beamish and McFarlane, 1983; Carlander, 1987). In addition, scales can be damaged or lost so regrown scales will also underestimate age (Zale et al., 2012). Nevertheless, if ageing is restricted to juvenile fish from higher latitudes most of these challenges can be avoided with proper care while sampling by taking a sufficient number of scales (i.e. at least 10 per specimen), from a location known to produce large, symmetrical scales, and from where they are less likely to be lost (Quist et al., 2007; Zale et al., 2012).

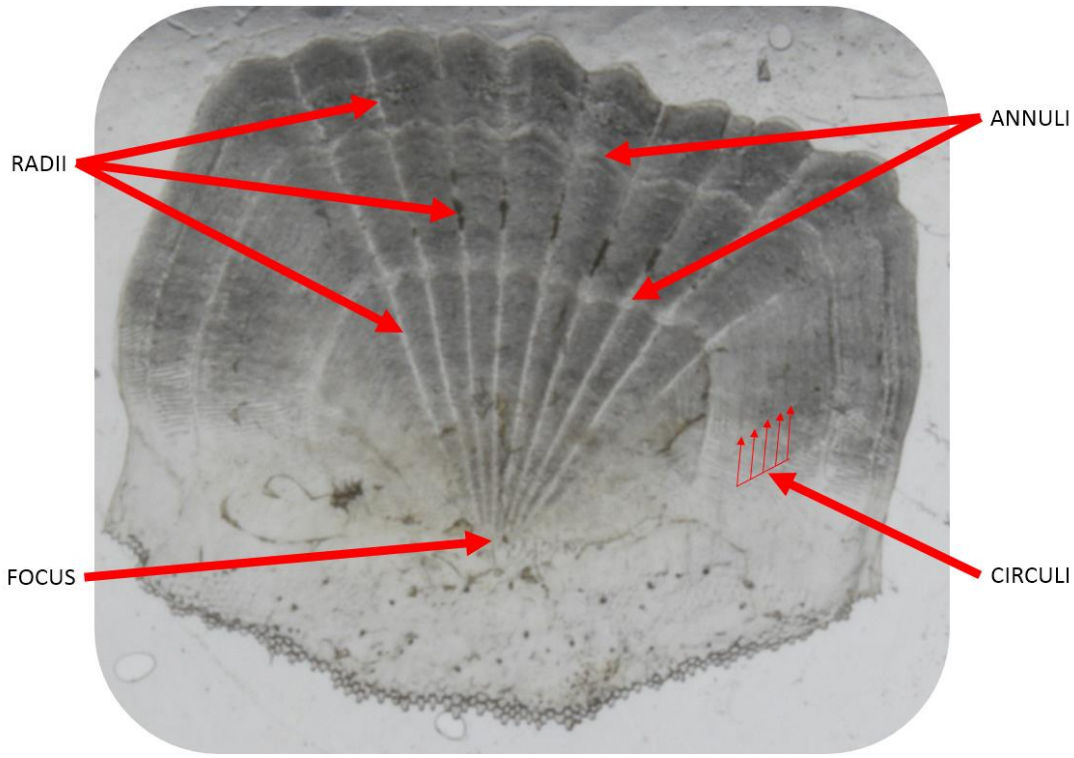

Figure 6.1. An example of snapper ctenoid scale (the type typically found on spiny-rayed fishes) with its features, taken during age and back-calculating past length determinisation. For a description see text.

6.1.3 Food-unlimited growth trials – means to investigate food limitation and maximum growth rates

Food is limiting for growth in the wild for some species and stocks (Jones, 1986; Le Pape and Bonhommeau, 2015) and it is not for others (Fonds et al., 1992, Boeuf and Payan, 2001). However, in many instances food as a nutrient and energy resource is highly related to seasonal dynamics and therefore it appears that only during less favourable times of the year food supply is not adequate for maximum growth (Jobling, 2008). In addition, when food was found to be limiting for fish growth outside its seasonal fluctuations, especially in recent times, it is often linked to major universal challenges such as global warming. The most frequently discussed topics are related to bottom-up effects via the impact on primary production (Boyd et al., 2007; Ward et al., 2012; Pedersen et al., 2014) that demonstrated declining propensities for the last few centuries (Boyce et al., 2010); overfishing of lower trophic level fish stocks on the top of already depleted stocks of many top predatory taxa (Hutchings and Reynolds 2004; Pauly et al., 2005; Cheung et al., 2010), or habitat degradation either of juvenile nursery grounds or deeper seafloor zones inhabited by commercially important demersal fish (Dobson, 2006). Despite efforts to reverse the trend, through means such as reductions in fishing efforts (Pauly et al., 2005) and increasing numbers and regions of marine protected areas (Edgar et al., 2014), the trend is still likely to continue for decades (Barnosky et al., 2016). Therefore, amongst other alternative approaches anthropogenic supplementary feeding of wild stocks as a relatively novel idea for stock enhancement has been developing and gaining encouraging evidence inviting further investigations (Björnsson, 2002, 2011; Björnsson et al., 2010).

The first necessary step when considering production enhancement of wild populations is to establish an argument whether the growth of the target fish stock is food limited (Björnsson, 2001). Another highly recommended guideline measure for studying fish growth and energetics is to gain data about maximum growth rates achievable under different environmental conditions (Jobling, 1994). In addition, one wants to know maximum potential growth rates of a prospective species to evaluate viability of its potential exploitation either for conventional aquaculture operations (Trushenski et al., 2012; Kailash et al., 2014), fish sea-farming or for a new-coming approach of anthropogenic feeding of wild stocks where information about differences between wild stocks growth rates and maximum potential growth rates will determine the level of economic attractiveness (Bjornsson, 2001; 2002; Halldersson et al., 2012). Obtaining maximum growth rates is commonly accomplished by feeding experimental fish to excess (Jobling, 1994). Exploring maximum growth capacity of wild stock scrutinised under unlimited food supply was an underlying principle employed by several authors whose findings revealed a conspicuous potential, in both a biological and an economic sense (Guderley et al., 1996; Björnsson 2002; Björnsson and Steinarsson 2002; Björnsson et al., 2010; Björnsson 2011). Guderley at al. (1996) demonstrated, by measuring biochemical growth

indicators (i.e. white muscle glycolytic and intestinal mitochondrial oxidative enzymes), seasonal fluctuations in food limitation for Atlantic cod (*Gadus morhua*) where the group of captured cod kept under full ration exhibited markedly higher growth rates and condition factors compared with wild data. This was evident during the period of low food availability (i.e. June) but the growth parameters were effectively the same between the two groups in October when food was more abundant in the wild (Guderley at al.,1996). Björnsson and Steinarsson (2002) were also using Atlantic cod to produce a model based on laboratory experiments to explain a relationship for estimating food unrestricted growth for a range of temperatures and fish sizes that can be used for evaluating growth performance in the natural environment as well as possibilities of cod farming. Their results indicated that overall cod growth in sampled areas of the North Atlantic was frequently food limiting. They extended their laboratory work to a wild setup where two tagged cod formations, one conditioned to anthropogenic feeding, and the second unconditioned, were followed for 17 months (Björnsson, 2002). Results showed a marked increase (~2 times) in growth rates of conditioned versus unconditioned fish. A further extension of the field experiment to a large-scale operation was reported in Björnsson et al. (2010) and Björnsson (2011) where supplementary feeding of conditioned cod additionally augmented all growth performance parameters showing over three times greater growth rates as a response, which emphasised a strong potential of this type of stock enhancement as a possible future remedy response to the conventional fishery crisis.

6.1.4 Snapper and YEM in New Zealand waters – are they food limiting in their natural habitats?

Snapper can be categorised into a group of fish whose growth is food limited at least during winter (Bell at al., 1991), when synergistic effects of two highly influential variables (food supply and temperature) cause growth to cease or even become negative (Francis, 1994; Cook et al., 2003). This scenario has been repeatedly noted for juvenile snapper; however, for adults, a density dependent competition for food resources was observed in addition to other growth constraints (Parsons, 2014). Besides, snapper larvae are especially susceptible to a lack of adequate food supply during the initial few months post-hatching as only well-built energy reserves permit survival over the first winter (Sim-Smit et al., 2013a). However, the typical pattern of food limitation effects on growth and survival of snapper as described here cannot be generalised and regarded as the same for all sites and habitats (Frances, 1994; Sim-Smith et al., 2013b). Food availability may differ between sites and seasons and therefore it is recommended that if growth traits are to be assessed in a new area that food limitation is also assessed (Frances, 1994; Sim-Smith et al., 2013b). There is no particular study conducted to explicitly attribute food limitation *per se* to growth of YEM, apart from Coubrough et al. (2004) who stated that food was likely a limiting resource in the wild. In addition, Webb (1973) reported a presence of food in YEM stomach all year around, suggesting that YEM in the Avon-Heathcote Estuary

do not starve even in winter. However, his aim was to qualitatively elaborate the content so no insights about food deficiency/limitation can be obtained from that study.

6.1.5 Objectives of the chapter

Food supply in its natural setting is one of the main factors affecting fish growth performance, but the direct impact of its limitation is often obscured by a combination of other factors such as temperature, oxygen or salinity levels. If food is limited in the wild, it is vital to understand what the extent of the limitation is or what is the difference between what can be achieved in the natural environments vs. what would be possible based on maximum growth potential of a species when food supply is not the growth limiting factor (ensuring all other conditions are equal). Based on that difference, estimates can be made if certain fish stocks are suitable for stock enhancement via for instance anthropogenic supplementary feeding or for fish sea-farming.

This chapter assumes that eliminating food constraints experienced in the wild via *ad libitum* feeding regime as utilised in the growth trials (chapter 2) will yield the best growth performance (i.e. maximum growth rates) and therefore be able to answer questions representing two main objectives of the chapter – is fish growth food limited in the wild and if it is how much is the enhanced (food-unlimited) growth greater in comparison to the one achievable in the natural environments.

The means of addressing these objectives were based on data generated in the food-unlimited growth trials (chapter 2) demonstrating what is possible when food is not limiting, and wild fish growth rate data which for snapper had two origins. First, data were defined by determining age and back-calculating past length at age via decoding growth marks imprinted on scales of wild-caught fish, and second, which also apply to YEM, the published data source. The final objective was to examine the effect of key abiotic (temperature) and biotic (food availability) environmental drivers on wild snapper growth rates that was achievable by examining satellite data depicting environmental fluctuation of the drivers in question over the corresponding past years.

6.2 Materials and Methods

To address objectives of this chapter first the focus was to gather wild fish growth data for comparison with the food-unlimited tank experiments, and subsequently to use these data to evaluate the outcomes of the growth study, as outlined in chapter 2. Snapper had two data sources that were used for this purpose. One was growth data derived from wild-caught fish in Tasman Bay, the South Island, New Zealand, where PFR research facilities associated with the present study are located and the other originated from published data from fish captured in the same area of the Bay where the wild-caught snapper originated (see Drummond and Kirk, 1986). YEMs only source of wild growth data was the published work of Curtis and Shima (2005).

The data related to the final objective (i.e. effects of food availability and sea-water temperature on wild snapper growth), namely data for sea surface temperature (SST) and chlorophyll *a* (a proxy for food availability), were obtained from several sources, which were originally derived from satellite data recorded for the past years, and these were backtracked to match with the growth periods of the wild caught snapper.

6.2.1 Wild snapper growth data acquisition – back-calculating past length at age from scales

The method for estimating the growth pattern of wild snapper was based on two sets of data. These were: wild snapper size measurements taken at capture (fork length and mass); and the calculation of age/length estimates from the annual increments imprinted on the calcified structure of scales. According to Paul (1976), scale ageing for *C. auratus* is considered valid for up to 10 years of age. Since a focus of this study was on juvenile fish (age of up to 4–5); this method was applicable for the wild snapper ageing. Wild fish for this purpose were sourced from the research trawl surveys conducted by PFR from 10 to 16 February 2015 in Tasman bay at four locations: Lat -41.101900, Long 173.175500 – Lat -41.114067, Long 173.187850, Lat -41.193900, Long 173.122567 – Lat -41.233067, Long 173.212083. The trawl locations, with a depth ranging from ~10 to 25 m were within a known juvenile ground, situated approximately 10 km from the PFR research facility where tank-based growth trials were conducted. The trawl vessel was equipped with precision seafood harvesting prototype fishing gear developed by PFR (PSH, http://www.precisionseafoodharvesting.co.nz/), as well as conventional four panel cod-end bottom/wing trawl gear. During the survey, juvenile and sub-adult snapper, mean size 185 mm fork length, were collected and transferred to the PFR facility, while kept live on board the trawl vessel in 250 L bins provided with a constant flow of seawater. On arrival

fish were lightly sedated in ~10 ppm AQUI-S and measurements for mass and fork length were taken with the approach as described in 2.2.3.3; however, scales were not taken during that measurement session. At the PFR facilities fish were held in 5000 L tanks at stocking densities below 7.5 kg 1000 L^{-1}. After approximately five months fish were remeasured (data utilised for generation of a correction factor only, see below) and scales were taken.

Since scales were taken 5 months post-capture, it was necessary to address the time discrepancy between immediate post-capture size measurements and scale collection before scale data were used for the purpose described above. This was addressed based on the assumption that fish scales grow proportionally with length growth (Ottaway and Simkiss, 1977; Schreck and Moyle, 1990; Zale et al., 2012). Factorial length growth, as a ratio of the length in July (i.e. 5 months post-capture) and the length on the day of capture, was determined for each fish and employed as a correction factor when it came to determination of age and back calculating past length at age. This was accomplished by reducing the measurement of a radius by the correction factor and considering the point of reduction as the edge of a scale at the time of the capture.

On the sampling day, 8 to 12 scales were removed from an area behind the left pectoral fin. Scales were labelled to identify the fish of origin and stored in paper envelopes until analysis. When processed, scales were placed on a microscope slide, wetted with nano-pure water, overlaid with a cover slip and imaged under a light microscope at 10–30 times magnification. The microscope used was a Nikon SMZ18 (Nikon, China) with a P2-FIRL LED ring illumination unit (Nikon, Japan) running on NIS-Elements D 4.30.00 (Build 1017) software. Once scales were imaged, images were analysed for age and back-calculation of past length and age. The image analysis was carried out, first with the Nikon NIS Elements D software for the initial scale sizing, and later with the Image*J* software (Image processing and analysis in Java, National Institutes of Health, Bethesda, Maryland, USA) for final analysis of scale ageing features.

The scale features required for analysis were focus, radii, and annuli. Annuli represent the point in time when fish are considered a year older and therefore are crucial for the age determination. However, the first annulus and conversely the distance from the focus to it does not necessarily characterise the time when the fish turns 1 or the growth of the fish within 1 calendar year respectively. The distance would normally represent less than 1 year of snapper growth, particularly for fish from Tasman Bay region. This is because the spawning season and reinstatement of snapper growth after winter may not perfectly match but rather lag time of a few months, between the two variables, may occur. The snapper spawning season is generally onset when the temperature threshold of ~15°C is reached, and it may extend from September to March, with the most active period being November–January (Crossland, 1977; Scott and Pankhurst, 1992; Francis, 1994b; Francis et al., 1995; Wakefield, 2010; Sim-Smith et al., 2013b). The triggering temperature for the Tasman Bay

populations is, on average, reached in mid-October (PFR monitoring station) and spawning peak is expected around December–January. Correspondingly, the fish used for the food-unlimited study (chapter 2) were hatched in mid-October 2013 and, according to Francis (1994a), the 1st of January was arbitrarily selected as theoretical birthday of wild-caught snapper.

Even though the first annulus on the scales of surveyed fish likely did not represent 1 year of age, but more plausible 10–11 months, the final difference in ages between the two populations (cultivated-experimental and captured fish) may not exceed more than 1–2 months. In addition, the comparison between the two populations was conducted in terms of annual fork length increase for a 12-month period, from age 1 to age 2. This corresponded with the growth pattern preserved as the distance between the first and second annuli representing the full calendar year of wild population fish development and growth. Moreover, the data for the assessment were accurately determined for the cultured fish via actual measurements taken exactly 1 calendar year apart, and for the captured wild fish by estimating yearly length increase via the annulus decoding procedure as explained earlier. Therefore, comparison between the food-unlimited Snapper population and the wild captured population was permissible.

6.2.2 Data manipulation: processing and calculations

By identifying annular markings on fish scales, the fish length at all annuli representing age can be back-calculated. For the calculation the following was required: fish length at capture, the scale radius at capture (estimated using the correction factor, see 6.2.1), and annulus increment radii (adjusted according to the correction factor).

Two approaches are proposed for back-calculating length at age:
The Dahl-Lea equation:

$$Li = Lc \left(\frac{Si}{Sc}\right)$$

where *Li* is the back-calculated length of the fish when an annulus formed, *Lc* is fork-length of fish at capture, *Si* is the annulus increment radius that corresponds with the *Li* (distance from the focus to the outer edge of the annulus in question), *Sc* is the radius of the scale at capture (distance along the groove from the focus to the outer edge of the scale). The Dahl-Lea equation is employed when the relationship between fish length and the scale radius has an intercept (a) that goes through the origin (zero), which means that the scale growth is directly proportional to body growth, since they begin to form immediately upon hatching. This equation is more commonly used when other hard structures like rays and otoliths are used for ageing, since in most instances when scales are the subject of age

and length at age determination the previously explained regression line does not pass through zero (scale growth begins later in early fish development, commonly at metamorphosis) then the Fraser-Lee approach is warranted (Zale et al., 2012). Since in the case of snapper a was above zero (i.e. 22 mm, compared to hatchery reared snapper at PFR facilities the metamorphosis occured at ~30 mm – Jerrett et al., unpublished data), the latter equation was employed for the calculations. The Fraser-Lee equation can be expressed as:

$$Li = \left(\frac{Lc - a}{Sc}\right) Si + a$$

where Li, Lc, Si and Sc are the same as for the Dahl-Lea equation and a is the intercept parameter of the above fish length/scale radius regression relationship.

6.2.2.1 Data manipulation: procedure

1. Descriptive statistics and the mass – length relationship (MLR) of growth data on the day of capture:

The entire wild fish data (fork length and mass at the capture) were pooled together for descriptive statistics, and as per chapter 2 methodology MLR was calculated. Wild stock MLR from all ages and separately from second year of growth were then compared with MLR from fish engaged in the growth study.

2. Construction of growth (length – age) curve of wild fish to be compared with the published and the growth study data:

Age was estimated by counting scale annuli and past length at age was back calculated based on the Frazer-Lee approach. The past length at age data were subsequently sorted into corresponding age groups (Fig. 6.2), so that all fish (age one and older) contributed to the mean fork length representing the first year of growth, fish age 2 and older contributed to the mean fork length in the second year of growth and so on. Finally, four fork length mean groups achieved in first, second, third and fourth year of growth of individual fish were derived and used to construct wild fish growth curve (Fig 6.2, 6.6C). The wild growth pattern was then compared with the published data for snapper captured in the same nursery ground area and the length-age curve constructed from only first two years of wild snapper data were compared with the growth study data.

3. Grouping yearly increments of back-calculated length data into corresponding year-classes:

The grouping proceeded in the way that the length growth for the first year of growth was divided into 2014, 2013, 2012 and 2011 year-classes (fish of all ages); the length increment for the second year of growth (i.e. the difference between fork length in second and first year) into 2013, 2012 and

2011 year-classes (fish age of 2+, 3+ and 4+), the length increment for the third year of growth into 2013 and 2014 year-classes (fish age 3+ and 4+), and fourth year of growth into 2014 year-class (fish age 4+ only) (Fig. 6.3, Table 6.1).
Such a grouping allowed for investigation into differences amongst the same age growth increments achieved at different calendar years. This also allowed testing how environmental conditions (see below) correlated with the variation in growth amongst those years.

6.2.3 Evaluation of environmental drivers modelling the growth of wild fish

Environmental drivers of interest were: abiotic – seawater temperature measured as surface seawater temperature (SST) and biotic – sea chlorophyll *a* concentration, as the index for phytoplankton biomass thus representing primary productivity as a base of the food chain in the selected area where the trawl surveys were conducted.

The oldest fish from the trawl survey exceeded four years of age. Therefore, to accommodate requirements for data from environmental drivers extended for at least 4 years since the survey, data from 01/12/2010 – 01/01/2016 was sought. Data for the environmental drivers were obtained from publicly available satellite datasets provided by ERDDAP – *easier access to scientific data* web page. Data for SST were sourced from the following link: http://coastwatch.pfeg.noaa.gov/erddap/griddap/jplG1SST.html, and data for chlorophyll *a* from http://coastwatch.pfeg.noaa.gov/erddap/griddap/erdMH1chla8day.html.

After downloading as an excel spread-sheet, all data extending over a calendar year spanning from 01/01 to 01/01 on the following year were averaged and descriptive statistics were calculated.

6.2.4 Statistical methods

Statistical tests:
The detailed description of statistical tests used in this chapter were explained previously in chapter 2 unless stated differently. Following are outlines of statistical objectives and tests carried out:

1. Testing for differences in fork length of the trawl survey growth data according to age (1 vs. 1, 2 vs. 2 etc.) amongst year-classes at age 1, 2 and 3 was carried out with one-way ANOVA and Student's t-test.

2. Comparison of absolute fork-length growth at age one and two and yearly fork length increase in the tank-based experiment data with the trawl survey growth data, was carried out with Students' t-test.

3. Producing the explanatory growth curve based on 3rd and 2nd polynomial function for the wild fish growth in all 4 years, and first 2 years respectively. The first 2 years' wild fish growth curve was used for comparison with the tank-based experimental data.

4. Calculating MLR, and relative mass (*Rm*) for the survey data. MLR between two populations was compared by extending the regression model (i.e. adding a factor *population*) as described in chapter 2 methods, and to compare relative conditions between the two populations relative mass in relation to mean mass (*Rm*) was utilised.

5. Relationship between SST and chlorophyll *a* was examined with the linear regression model.

6. The correlation test was employed to investigate the association between STT and fork length of wild fish in the first and second year of growth and the same was carried out for the chlorophyll *a* dataset. Since the correlation test can practically be performed only with the minimum of three data pairs, correlation between environmental drivers and length means for 2014 and 13 year-classes was not appropriate as the numbers of data pairs were one and two respectively.

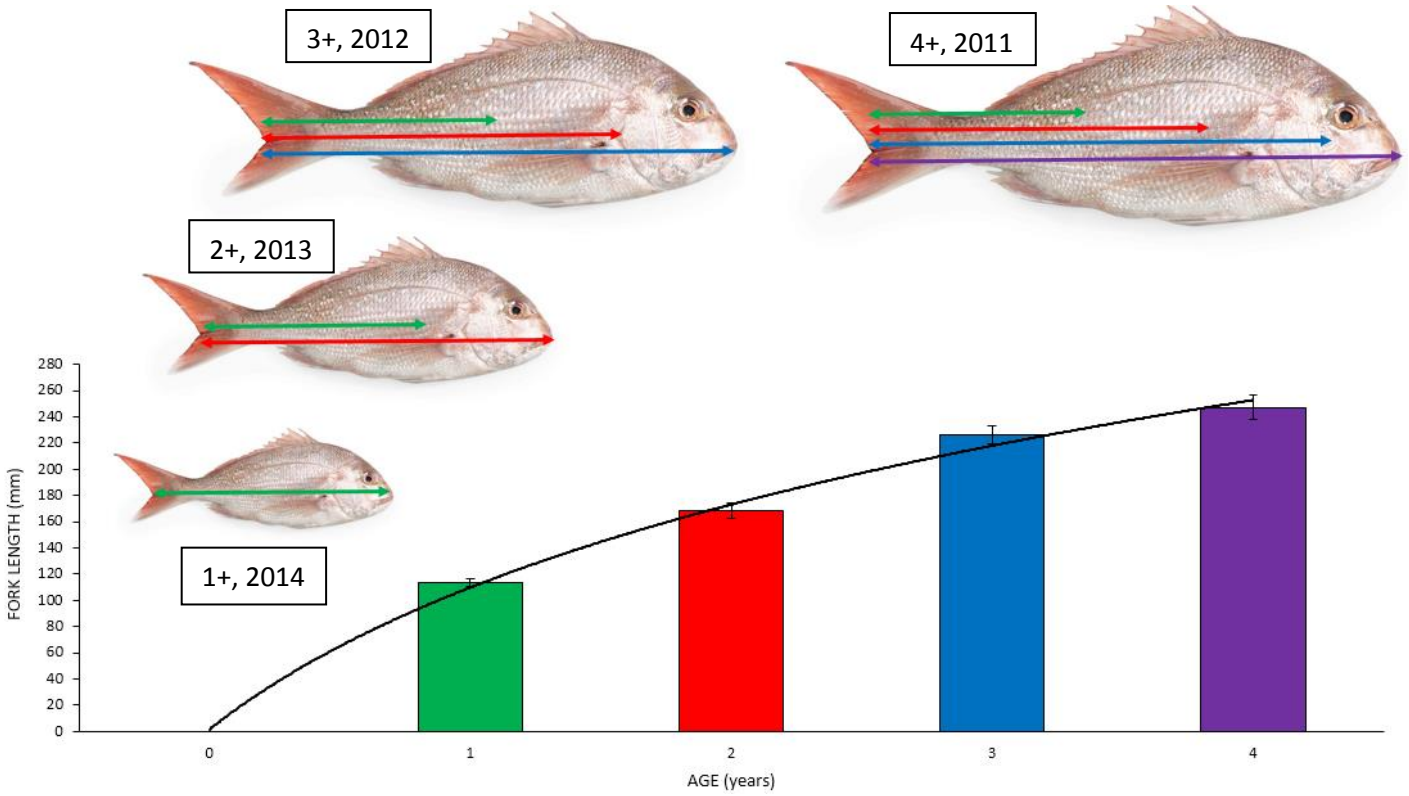

Figure 6.2. Colour coded illustration of grouping of estimated back-calculated fork length values into age groups (green bar – mean fork length at the age 1, red bar – mean fork length at the age 2, blue bar – mean fork length at the age 3 and purple – mean fork length at the age 4). The colours of bars correspond with colours of double-arrowed vectors. Numbers in boxes next to fish images (i.e. 1+, 2+, 3+ and 4+) are estimated fish age which correspond with the colour and the length of longest or only vector on fish images, and years in boxes are year-classes. Black curve is natural logarithmic function fitted to the growth data. Error bars are 95% confidence intervals.

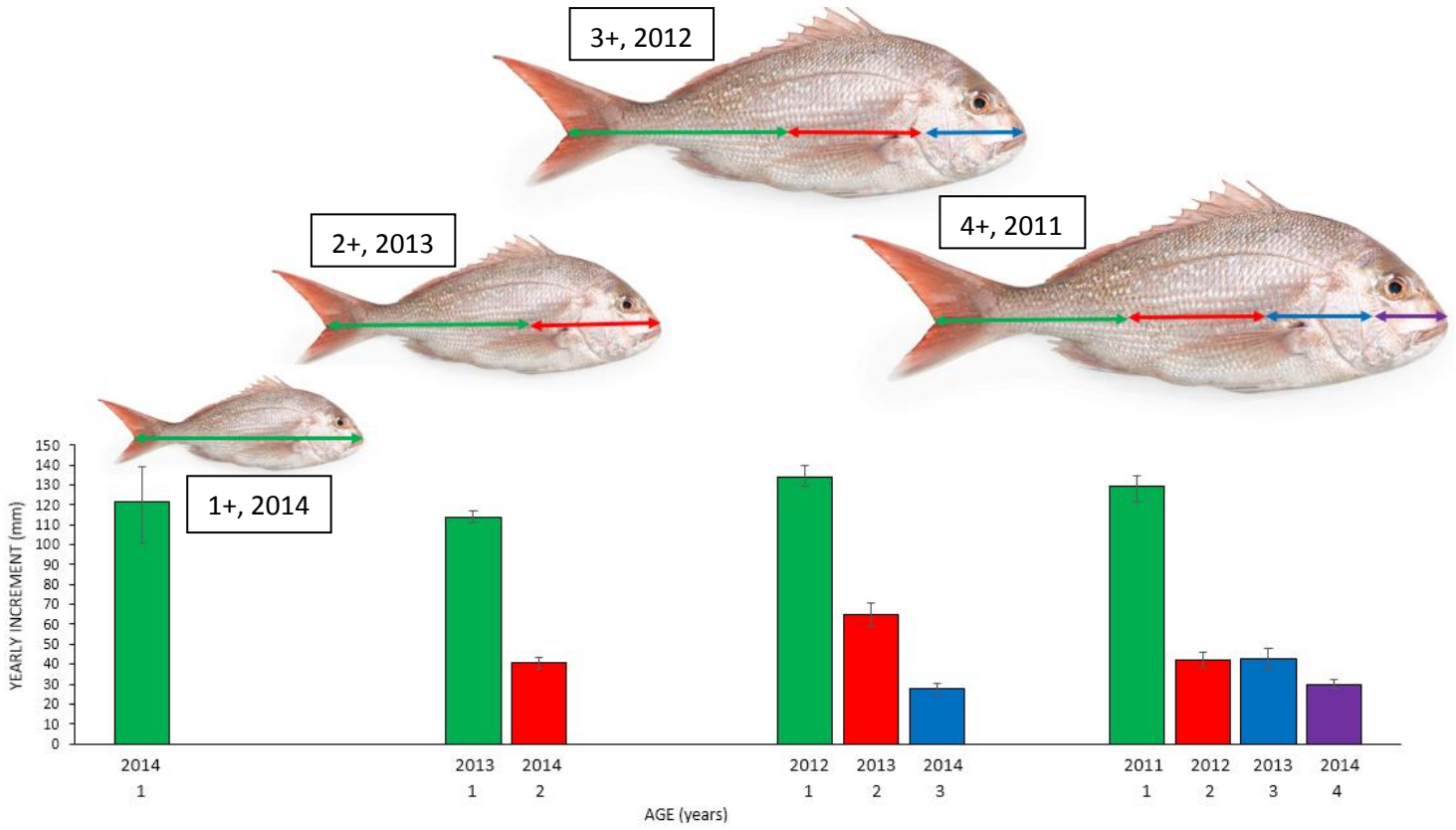

Figure 6.3. Colour coded illustration of grouping of estimated back-calculated length increments into year-classes (green bar – mean length increment at the age 1, red bar – mean length increment at the age 2, blue bar – mean length increment at the age 3 and purple – mean length increment at the age 4). The colours of bars correspond with colours of double-arrowed vectors. Numbers in boxes next to fish images (1+, 2+, 3+ and 4+) are estimated fish age which correspond with the colour and the position of far-right or only vector on fish images, and years in boxes are year-classes. Error bars are 95% confidence intervals.

6.3 Results

6.3.1 Snapper – back-calculated past length at age from scales

6.3.1.1 Wild snapper growth – effects of years and age

Wild fish length growth data were organised according to age determined by decoding the scale markings into year-classes 2011, 12, 13 and 14 when the first growth year took place. Descriptive statistics (means and standard deviations) of back-calculated past lengths at age and annual length increments for four year-classes are outlined in Table 6.1.

The greatest fork length growth achieved in the first year of growth amongst year-classes was observed for the 2012 class (Table 6.1, Fig. 6.2). The significantly lowest growth was the 2013 class (2012 vs. 2013, $t = 6.538$, $p < 0.001$), while the only other difference amongst year-classes was detected between 2011 and 2013-year fish ($t = 4.661$, $p < 0.001$, Table 6.4). The second year of growth of surveyed fish also varied between year-classes where the highest annual length increment was observed for 2012 year-class (Table 6.1), which was higher than annual increase for both 2011 ($t = 5.867$, $p < 0.001$) and 2013 year-classes ($t = 8.335$, $p < 0.001$). The third year of growth was higher in 2012 year-class compared to 2011 ($t = 5.756$, $p < 0.001$), which made fish hatched on transition from 2011 to 2012 the highest growing class for the first and second year growth amongst all four surveyed age fish groups, but not for the third-year growth which was the highest for the 2011 year-class juvenile snapper (Table 6.1, Fig.6.4).

6.3.1.2 Fork length growth, wild vs. experimental fish

When all wild first year length growth data were pooled together ($n = 88$, Table 6.1) and compared via Student's t-test with the cultured snapper data ($n = 312$) obtained at the time when they entered the experimental protocol (both groups at age ~ 1 year) there were no differences observed (Mann-Whitney Rank Sum Test U statistics = 12272.50, $p = 0.172$). However, when the same experimental data were compared with the first-year growth for 2012 class fish (the group with the highest first year annual increase), it was evident that wild fish were bigger than the cultured group ($t = 6.850$, $p < 0.001$, Table 6.1). Furthermore, the rank sum test employed for comparison between experimental ($n = 81$) and all wild fish fork length data at age ~2 ($n = 84$, Table 6.1) revealed that significantly bigger fish belonged to the experimental cohort ($U = 1077.00$, $p < 0.001$), although a comparison of the experimental and 2012-year class data showed no difference ($t = 0.037$, $p = 0.970$). When annual

growth increment rather than absolute length growth was used as the comparison parameter, experimental fish showed the greater increment than both the pooled wild data and the 2012-year class ($t = 14.539$, $p < 0.001$; $t = 4.006$, $p < 0.001$, respectively; Fig. 6.5).

6.3.2 Length-age curve of wild fish, experimental fish and published data

Experimental and wild data for the first 2 years of growth are graphically portrayed in Figure 6.6A. Both sets of data as a function of age were explained with 2nd polynomial function [i.e. length (mm) = -19.426*(age in years)^2 + 138.35*(age in years) + 1E-13, $R^2 = 1$; and length (mm) = -37.374*(age in years)^2 + 158.49*(age in years) + 1E-13, $R^2 = 1$, for experimental and wild fish respectively]. Polynomial function fitted to the growth data suggested that the deviation in the growth direction started to be obvious at the point when the food unlimited growth trial began (~age 1), while until that point the trajectory could be regarded the same (Fig. 6.6A). When these data were compared with data extrapolated from a length – age curve (Fig. 6.6B) for Tasman Bay/Golden Bay snapper from the 1984/85 juvenile snapper survey published by Drummond and Kirk (1986), it appeared that fork length values from 1984/85 survey length –age curve were the highest relative to experimental and wild fish (PFR February 2015 survey) for both the first and the second year of growth (Table 6.1). Figure 6.6C depicts back-calculated mean fork length at age for 2015 juvenile snapper survey data fitted to 3rd polynomial function [length (mm) = 3.593*(age in years)^3 - 34.945*(age in years)^2 + 143.66*(age in years) + 1.7611, $R^2 = 0.994$], which well represented growth at all four age groups. The Drummond and Kirk (1986) curve encompassed juvenile fish between 1 and 3 years, therefore it allowed for observational mean comparison with wild fish data for age 1 to 3 (Fig. 6.6B, C). Once again, 1984/85 survey snapper fork-length mean was higher than for wild fish data (Table 6.1).

Table 6.1. Mean and standard deviation annual length increment (mm) and back-calculated lengths at age for four year-classes of snapper captured in Tasman Bay during the February 2015 PFR juvenile snapper survey. The last three rows are overall mean and standard deviation back-calculated length at age for the surveyed fish, estimated fork length values from length-age curve for Drummond and Kirk (1986) juvenile snapper survey, (Fig. 6.6B) and mean and standard deviation for food–unlimited experimental fish.

			Mean and standard deviation annual length increment at age (mm)							
			1		2		3		4	
Age	Year-class	n	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	2014	4	121.49	15.69						
2	2013	50	113.96	10.30	40.65	10.39				
3	2012	19	134.03	12.55	64.97	12.72	28.03	4.36		
4	2011	15	129.59	10.39	42.15	6.82	42.52	10.21	29.9	4.71
Overall mean and SD annual length increment			121.11	14.16	46.38	14.32	34.62	10.48	29.9	4.71

			Mean and standard deviation back-calculated lengths at age (mm)							
			1		2		3		4	
Age	Year-class	n	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	2014	4	121.49	15.69						
2	2013	50	113.96	10.30	154.61	16.18				
3	2012	19	134.03	12.55	198.82	21.98	226.85	21.06		
4	2011	15	129.59	10.39	172.79	13.02	215.31	12.23	245.21	14.85
(n)			88		84		34		15	
Overall mean and SD back-calculated length at age			121.11	14.16	167.48	24.72	222.29	19.13	245.21	14.85
Drummond and Kirk (1986)			132		206		239			
Experimental data			118.93	9.07	199.01	17.74				

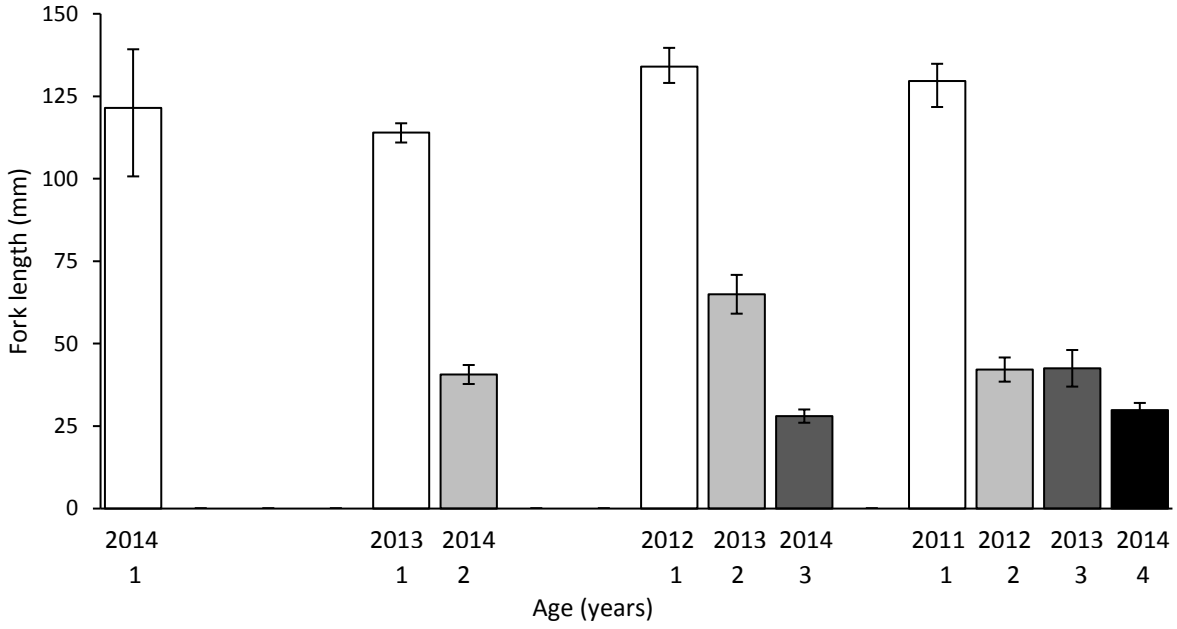

Figure 6.4. Mean values of annual back-calculated fork length increments at age (second bottom raw numbers from 1 to 4) of wild snapper surveyed in February 2015 in Tasman Bay (for details see 6.2.1). White bars represent first year of length growth of corresponding year-classes, light grey bars represent second year length increments, dark grey third and black fourth. Error bars are 95% confidence intervals.

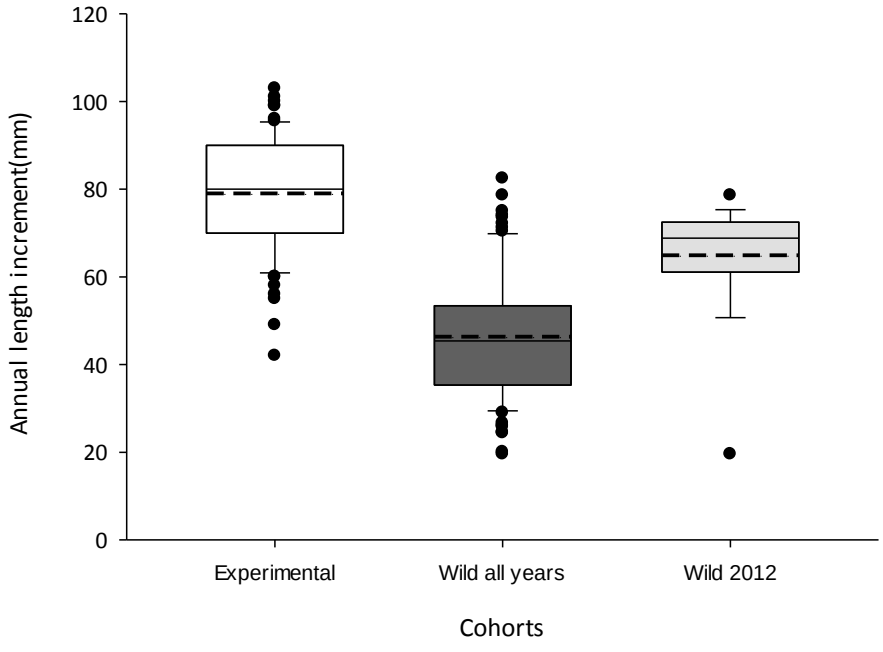

Figure 6.5. Box plot presentation of mean values of annual back-calculated fork length increments for the second year of growth for experimental fish (white box), wild surveyed fish data pooled from all available year-classes (dark grey box) and wild fish for 2012 year-class only (light grey box). The ends of the boxes define the 25th and 75th percentiles, with a middle line at the median and error bars defining the 10th and 90th percentiles. Black dots are data points outside of the 10th/90th percentiles envelop. Dashed lines within boxes represent mean values.

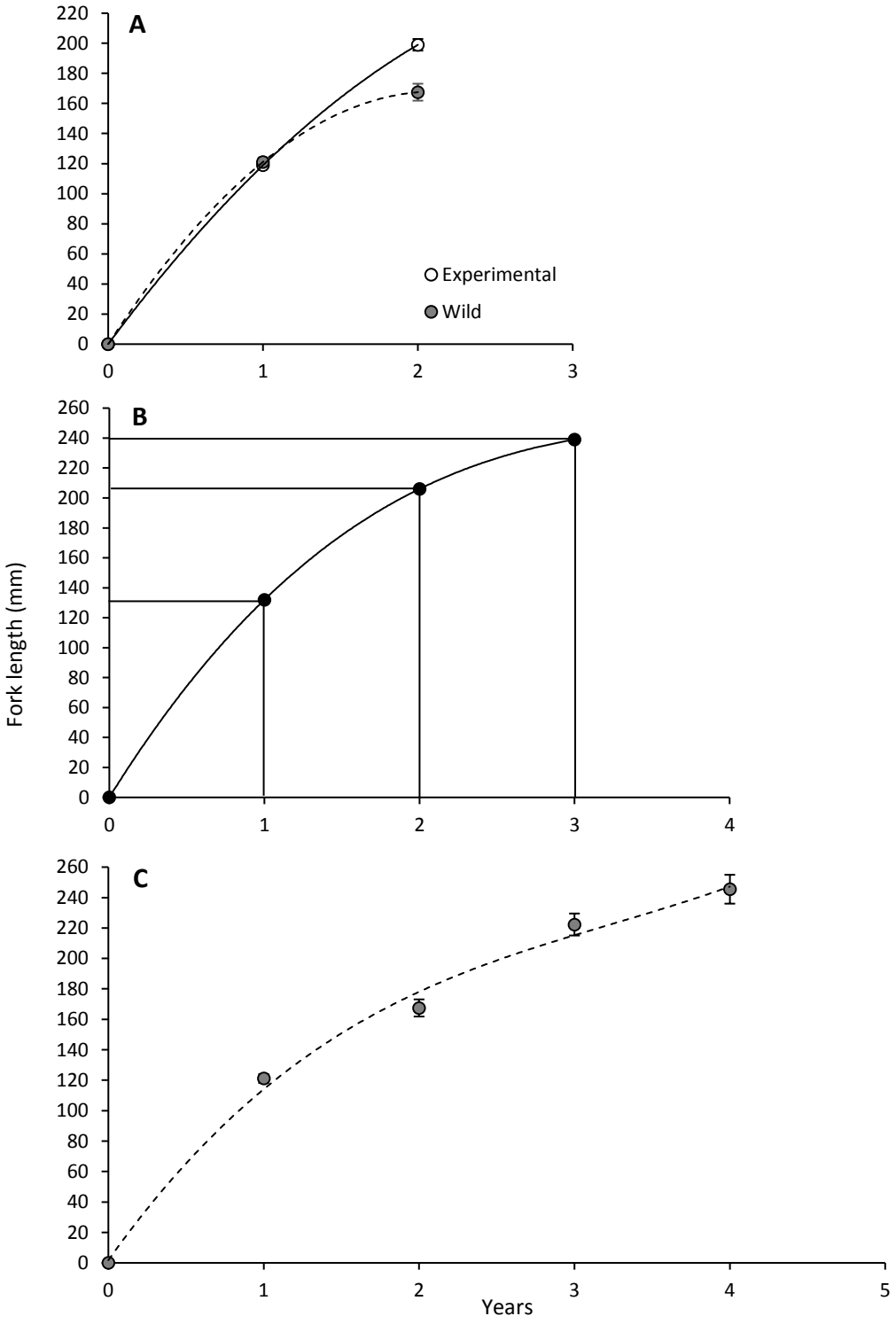

Figure 6.6. The relationship between snapper age and fork-length in three scenarios as described in text: A – means of 2 years of growth for experimental (black curve with open symbols) and wild snapper (PFR February 2015 survey, dashed curve with grey symbols) data fitted to 2nd polynomial function; B – 3 years of growth for Tasman Bay/Golden Bay snapper from 1984/85 juvenile snapper survey (curve reconstructed from data acquired from Drummond and Kirk, 1986) with added vertical and horizontal black lines assisting in length determination with the corresponding age; C – means of 4 years of growth for wild snapper (PFR survey) data fitted to 3rd polynomial function (curve). Error bars are 95% connivance intervals.

6.3.3 Wild fish mass – length relationship (MLR) and relative mass (*Rm*)

6.3.3.1 Wild fish MLR and comparison with experimental data

On the day when the 2015 juvenile survey was carried out all fish were measured for mass and fork length. The data were utilised for determination of MLR, which can be expressed in an arithmetic (i.e. two parameter power model) form: $M = 0.00002\ L^{3.028}$ and in a base-10 logarithmically transformed form with the equation of the best fit ($R^2 = 0.99$) as $\log_{10}(M) = -4.710 + 3.028 * \log_{10}(L)$ with 95% confidence limits for a = -4.805 – -4.615, and b = 2.986 – 3.070. The range of the confidence limits indicated overall isometric growth of surveyed snapper since b exponent value of 3 was included within the limits. When the entire data used for overall MLR assessment were arranged according to age, and separate MLRs were calculated, it became apparent that the relationship was not balanced for all four wild age groups as exponent b from the arithmetic form of the relationship demonstrated (i.e. n = 4, $R^2 = 0.74$, b = 3.238; n = 38, $R^2 = 0.97$, b = 3.147; n = 14, $R^2 = 0.81$ b = 2.630; n = 13, $R^2 = 0.95$, b = 2.644 for age 1, 2, 3 and 4 respectively).

When combined wild fish MLR data were compared, as described in chapter 2 Methods, with entire experimental MLR data a difference was observed which specified that on the plot with the log10 transformed data significantly more mass growth occurred for every unit length increase for experimental fish relative to the wild surveyed group ($F_{1, 928} = 375.95$, $p < 0.001$, Fig. 6.7). However, since experimental data encompassed 12 months (all seasons) of repeated measurements on the same individuals and the wild data were obtained in February only, the comparison between the two data sets should be regarded only as informative. Therefore, more relative comparison between February 2015 experimental data and wild fish February data, when both groups were at the same 1+ age, was required. This was not entirely possible since only four wild fish were found to match the aforementioned criteria. This would make the comparison of the two relationships inadequate since probability of committing a type II error (i.e. not detecting a difference when the one in true exists) would be markedly increased. The closest arrangement was to combine 1 + (2014 year-class) with 2 + (2013 year-class) wild age groups together and compare their MLR with experimental February data. This comparison revealed the same trend previously observed, that on the plot with log10 transformed data for the month of February experimental fish had greater mass increase with increase in length than wild cohort ($F_{1, 110} = 3.89$, $p = 0.048$, Fig. 6.8).

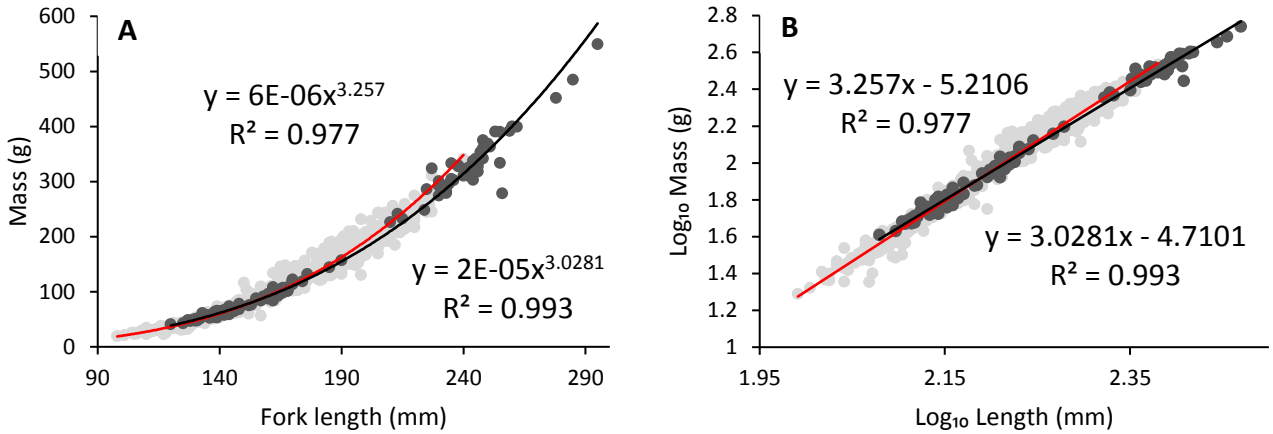

Figure 6.7. A – All experimental (light grey symbols and red curve) and wild surveyed snapper (dark grey symbols and black curve) mass-length relationship data fitted to power model (curves) with associated equations and R^2s. B – log_{10} base transformed mass-length relationship data for experimental (light grey symbols and red line) and wild fish (dark grey symbols and black line) from figure A, fitted to linear model with associated equations and R^2s (lines).

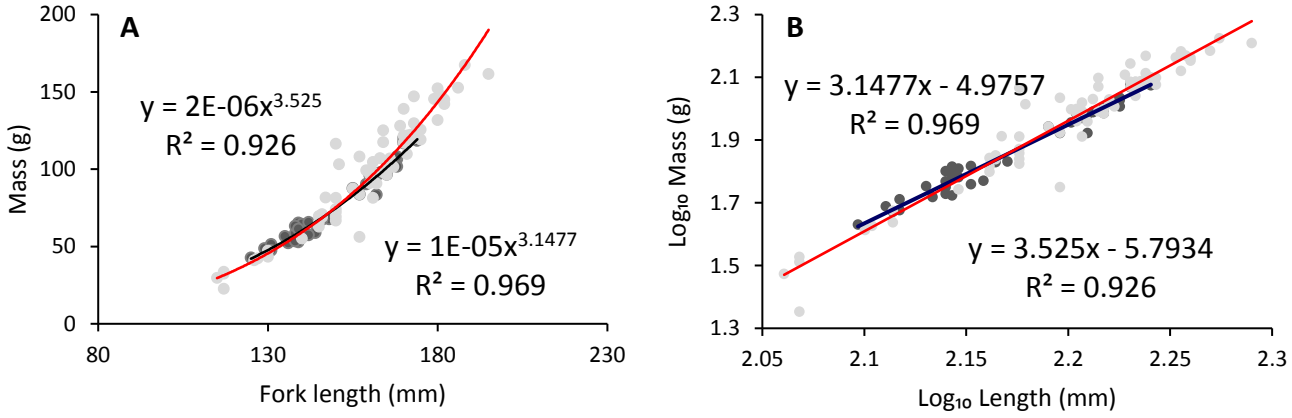

Figure 6.8. A – February experimental (light grey symbols and red curve) and 2013 and 14 year-class wild surveyed snapper data (dark grey symbols and black curve). The rest of the caption is the same as for Fig. 6.7.

6.3.3.2 Relative condition – relative mass in relation to mean mass (*Rm*) and comparison with experimental data

Relative condition (%) expressed as relative mass (*Rm*) for entire wild sample (mean = 87.0% ± 5.6 S.D., n = 4) when compared with experimental snapper *Rm* (mean = 102.3% ± 6.2 S.D., n = 11) was

significantly lower as Mann-Whitney Rank Sum Test revealed (U test statistic= 4379.00, $p < 0.001$). In an analogous manner to 6.3.2.1, more acceptable condition comparison was required. Therefore, implementing the same principle described in 6.3.2.1 the additional comparison procedure was performed which demonstrated that the *Rm* of experimental fish for the month February 2015 was higher than for the wild fish when data from 1 + (2014 year-class) with 2 + (2013 year-class) were pooled together ($U = 115.00$, $p < 0.001$).

6.3.4 Environmental drivers of growth – correlations with survey fish data

Satellite sea surface temperature (SST) and chlorophyll *a* data for the region where the trawl survey was conducted in February 2015 were submitted to regression testing and results demonstrated a strong negative correlation between these two environmental drivers for years spanning from 2011 to 2014 ($R = 0.978$, $R^2 = 0.956$, $F_{1, 3} = 43.71$, $p = 0.022$, Fig. 6.9).

To address the question whether two main environmental drivers associated with growth parameters of snapper, SST and chlorophyll *a* data were tested for correlation strength, direction and significance level with wild snapper fork length data. When length data for the first year of growth were regressed against SST data negative correlation was revealed ($R = 0.891$, $R^2 = 0.795$, Fig. 6.10A). However, significance levels were not reached, primarily because of the small number of regression pairs (four) involved in the statistical procedures ($F_{1, 3} = 7.74$, $p = 0.109$). Similarly, Chlorophyll *a* data was highly positively correlated with wild snapper length data that for the same reason turned out not to be significant ($R = 0.904$, $R^2 = 0.818$, $F_{1, 3} = 8.97$, $p = 0.096$, Fig. 6.10B).

In contrast to the first year of growth in terms of direction of correlation between wild snapper fork length data and the two environmental drivers, second year of growth had again obvious association with both SST and chlorophyll *a* but with no significance level observed, nevetheless the correlation was positive with SST and negative with chlorophyll *a* ($R = 0.975$, $R^2 = 0.951$, $F_{1, 3} = 19.34$, $p = 0.142$; $R = 0.941$, $R^2 = 0.885$, $F_{1, 3} = 7.72$, $p = 0.220$; for correlation with SST and chlorophyll *a* respectively, Fig. 6.11A, B).

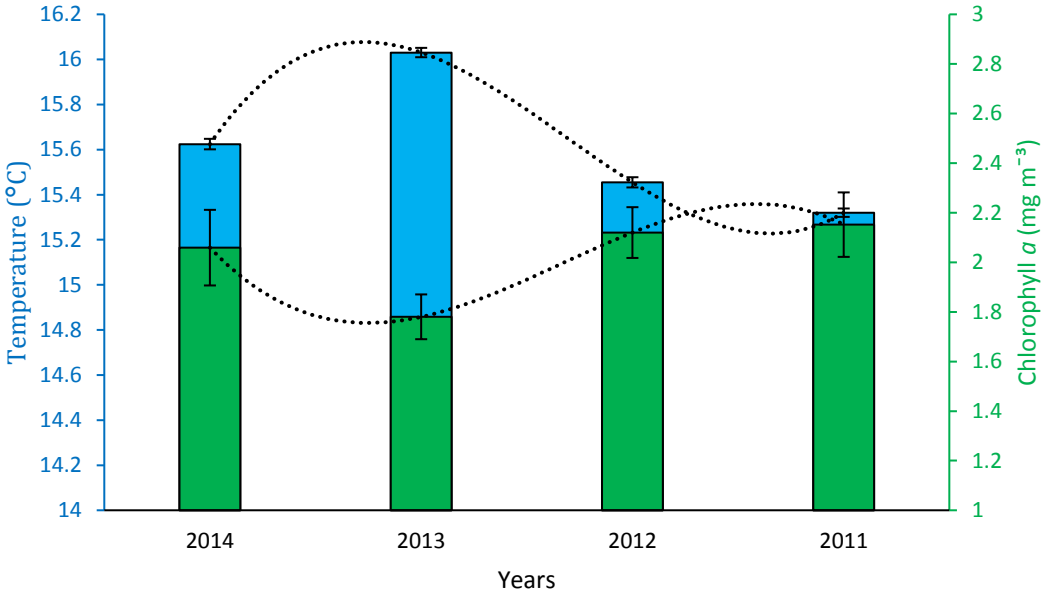

Figure 6.9. Satellite mean sea surface temperature (SST, blue bars) and chlorophyll *a* (green bars) over a course of 4 years (2011 – 2014) for the area of Tasman Bay where the February 2015 trawl juvenile snapper survey was conducted (for GPS coordinates see 6.2.1). Black dotted curves are 3rd order polynomial function fitted to the data for the illustration purposes. Bars are 95% confidence intervals.

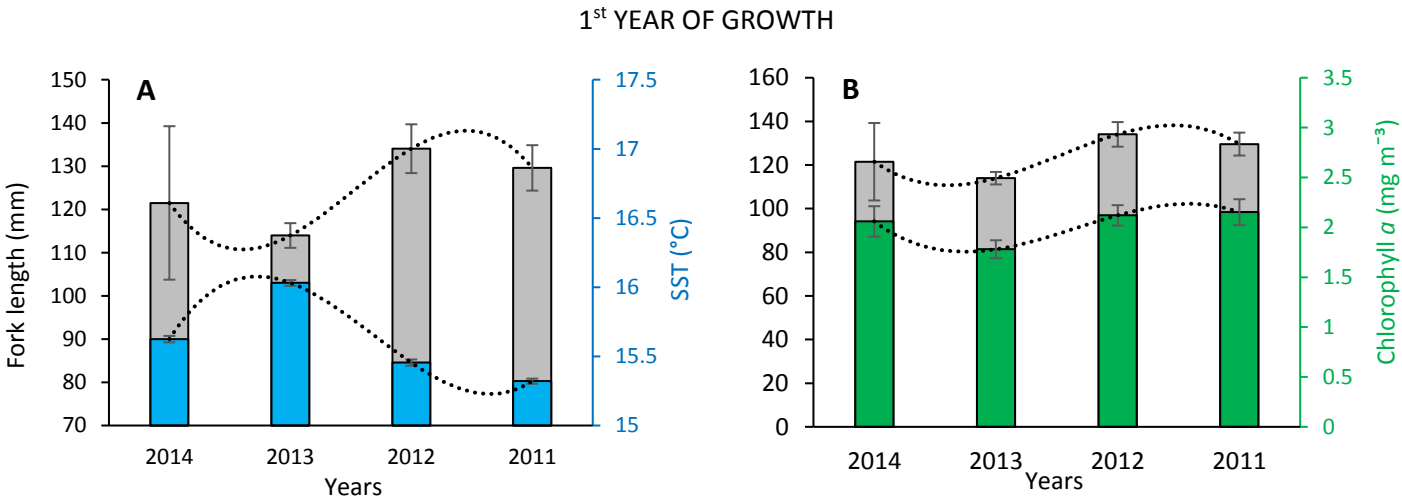

Figure 6.10. Mean fork length of first year of growth for all four year-class surveyed snapper (light grey bars) and SST satellite data (blue bars) – A, and chlorophyll *a* satellite data (green bars) – B; for the region where the survey was conducted. Black dotted curves are 3rd order polynomial function fitted to the data for the illustration purposes. Bars are 95% confidence intervals.

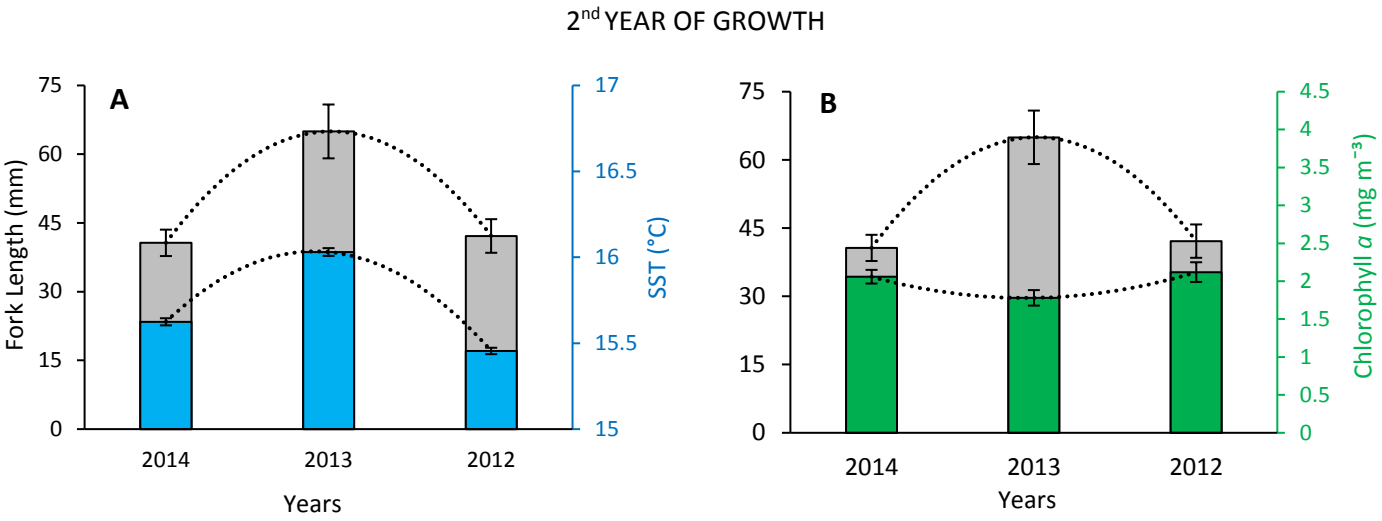

Figure 6.11. Mean fork length of second year of growth for 2011, 12 and 13 year-classes surveyed fish (light grey bars) and SST satellite data (blue bars) – A, and chlorophyll *a* satellite data (green bars) – B, for the region where the survey was conducted. Black dotted curves are 2nd order polynomial function fitted to the data for the illustration purposes. Bars are 95% confidence intervals.

6.3.5 YEM growth comparison with growth data published by Curtis and Shima (2005)

Curtis and Shima (2005) conducted a comprehensive nationwide YEM survey that included 14 selected estuaries and harbours around New Zealand to investigate geographical and sex-specific variation in YEM growth dynamics. Geographically the most related location to the area where the growth study YEM originated from (only ~50 km air distance between the two sites) was the Havelock area of the Marlborough region, North South Island (Fig. 6.12). Therefore, data from that region were utilised to address requirements of the present study. Firstly, Curtis and Shima's data were used for age determination of YEM used in the growth experiment, and secondly for annual growth increment comparison with wild fish to asses YEM growth capacity when submitted to *ad libitum* feeding regime. Since Curtis and Shima (2005) used total length measurements (the length taken from the tip of the snout to the furthermost end of the caudal fin) rather than fork length, as practiced in this study, a correction factor was generated (1.072) and utilised to bring the YEM growth experiment data in agreement with data from Curtis and Shima (2005). YEM entered the growth experiment at average fork length of 140 mm or ~150 mm total length, which corresponded with age of 1.8 (~1 year and 10 months) according to Curtis and Shima (2005). Furthermore, estimated total length of wild YEM 1 year later (age ~2.8) was 215 mm. Experimental mean YEM fork length from the end of the growth study (244.6 mm) were converted into the total length measurement of 262.2 mm. The difference

between the two lengths at age ~2.8 was ~50 mm. This indicated ~22% greater length increase in one calendar year achieved for YEM in food-unlimited experiment compared with wild fish data.

Figure 6.12. A map of north of the South Island with highlighted Nelson and Havelock connected with a red double-arrow vector as a ~50 km air distance between the two sites. In Nelson Haven, which is Maitai River estuary, YEM for the food-unlimited growth experiment were caught; and in Havelock region where an estuary of Pelorus River is located, was one of the sites for Curtis and Shima (2005) nationwide YEM survey.

6.4. Discussion

6.4.1 General observation

Wild snapper length growth data demonstrated interannual variability within year classes spanning from 2011 to 2014. This indicated differences in year class strength (YCS) as a reflection of interannual fluctuations in persuasive environmental variables such as sea-water temperature and food supply. Compared with experimental fish at age ~2, wild snapper at the same age grew less in length, but this was only true for all year classes when annual growth increment was considered. Three-way comparison of experimental/wild/published data revealed that Drummond and Kirk (1986) juvenile snapper was the largest of the three groups both at ~2nd and ~3rd year of age; however, their results may be overestimated. In terms of growth type based on MLR (mass/length relationship) wild fish exhibited overall isometric growth, whereas the experimental group grew hyperallometrically indicating that greater mass increase with increase in length was prominent for the experimental cohort than a wild one. Likewise, experimental fish condition was greater than in wild fish. Therefore, it appears that food could be in short supply, limiting growth and affecting condition and fitness of juvenile snapper in Tasman Bay, New Zealand. In comparable fashion, experimental YEM demonstrated greater length growth relative to wild fish, similar to snapper, suggesting that food availability may be limiting in natural environments.

6.4.2 Snapper – spatial and temporal variation in growth performance

Australasian snapper *C. auratus* have a biogeographical extent ~15–40° S (Crossland, 1981; Paulin, 1990; Gomon, 2008) occupying a variety of depths (10–200 m; Francis, 1993) and substrate niches ranging from soft-sediment coastal habitats to rocky reefs, coralline turf algae and sand flats (Francis, 1995; Ross et al., 2007; Compton et al., 2012). They are opportunistic predatory feeders capable of utilising a variety of food items and adjusting their feeding strategies according to prey availability (Godfriaux, 1969). Keeping those facts in mind, it can be expected that snapper would exhibit distinct variability in growth performance on both spatial and temporal scales.

6.4.2.1 Spatial variation in wild snapper growth performance

Spatial variations in growth characteristics of snapper and related sparids have been found over large latitudinal and longitudinal distances (Francis, 1994a; Potts and Manooch, 2002). But

differences in snapper life history traits were also evident on a much smaller scale, as been observed in Western Australian populations where within less than 10 km, differences were evident (Jackson et al., 2010). The suggested explanation pointed to dissimilarities in environmental conditions as well as fishing-induced density dependent responses (Jackson et al., 2010). Snapper growth rates were also found to vary considerably amongst New Zealand sites (Francis, 1994a). For example, the Tasman Bay population was found to grow faster than fish in Hauraki Gulf (the North Island), but since the differences in growth rates (GR) were not detected before 2–3 years of age, the early snapper growth appeared to be similar amongst major New Zealand juvenile sites (Francis, 1994a). Commonly, growth data from wild fish is represented with length measurements; however, occasionally they are accompanied with mass data and by the relationship between the two, a reliable stock assessment can be carried out (Froese, 2006). The slope b of MLR informs about the character of the growth, which can be isometric, hypoallometric or hyperallometric when b is equal, below or above 3.0, meaning that all fish dimensions increase at the same rate, fish increase in mass less than predicted by their length growth, or they increase in mass more than predicted by their length respectively (Froese et al., 2011). MLR is therefore also expected to vary spatially. In this study, overall MLR indicated isometric growth of wild juvenile snapper from Tasman Bay. When comparing Tasman Bay and Hauraki Gulf MLR, knowing that fish grow faster in the former, it is not surprising that parameter b of the Hauraki Gulf MLR was often found below 3.0, indicating hypoallometric growth (e.g. 2.80–2.90 in Morrison and Francis, 1999; Maunder and Starr, 2001 and Majed et al., 2002b). Yet, Francis (1997) reported for the same snapper population for the period extending from 1985 to 1990 all three types of growth designated with b value from 2.91 to 3.19. Similarly, in the only other published MLR from the Tasman Bay/Golden Bay area by Paul (1976), negative allometric growth (b = 2.793) was found. This discrepancy in the growth type for the same locations but different years demonstrates temporal effects on snapper growth performance.

6.4.2.2 Temporal variation in wild snapper growth performance

Based on back-calculated past length at age from fish scales, wild snapper growth data showed annual differences in growth performance within year classes. Often annual variability in population age and size structure are expressed as year-class strength (YCS) which includes both abundance of fish and their sizes (Fowler, 2002; Fowler and Jennings, 2004). In several previous studies using *C. auratus* as a model, an annual environmental variable that exhibited the strongest correlations with the YCS was sea-surface temperature (SST). In Hauraki Gulf snapper, Francis (1993, 1994a) and Francis et al., (1995) demonstrated that 94, 25–77 and 89% of variability in 1+ year class respectively, was explained by SST of the previous year (0+ year). Accordingly, Francis (1993) concluded that there was a strong, direct or indirect underlying relationship between YCS and SST governing the link

between the two variables. Besides, the three studies suggested a strong predictable power of SST in estimating 1+ snapper YCS for the Hauraki Gulf. However, Fowler and Jennings (2004) did not find a clear relationship between 1+ YCS and temperature in the South Australian snapper population for the period between 2000 and 2002 where variability in YCS was present but did not vary as strong as would be expected based on SST. Therefore, they suggested that there was not a simple linear relationship between strength of recruitment/YCS and SST, indicating that regulatory processes in the Spencer Gulf, South Australia, may differ to those observed in the Hauraki Gulf (Fowler and Jennings, 2004).

In the present study an objective was to investigate direct effects of SST and net primary production (NPP) on juvenile snapper growth performance for the given year of growth rather than to follow the practice of aforementioned studies. Nevertheless, in line with Fowler and Jennings (2004), data from the present study, although not addressing the predictability of 1+ YCS by SST, may suggest that the interannual regulatory processes determining strength of YCS between Hauraki Gulf and Tasman Bay may also differ. Data from the present study, in contrast to those reported for Hauraki Gulf and South Australia where SST positively affected YCS, showed negative correlation between SST and growth performance of 1+ juvenile snapper and a concurrent positive correlation with the proxy for net primary production (NPP) or chlorophyll *a* concentration. The paramount importance of available NPP for potential marine fisheries production has been recognised in many studies (e.g. Boudreau and Dickie, 1992; Ware and Thompson, 2005; Chassot et al., 2010). Furthermore, Blanchard et al., (2012) proposed that the change in fish productivity and growth in large marine ecosystems, and likely globally, is evidently reflected by interannual variability in phytoplankton production. Therefore, NPP could be used as a proxy for fish growth since it is more often and more positively correlated with an increase in fish biomass than SST, indicating that the relationship between NPP and SST is generally complex (Arrigo et al., 2008). For example, in the Southern Ocean the association between the two variables was found to vary from positive to negative (Arrigo et al., 2008). SST is believed to positively affect annual primary production because phytoplankton growth rates are increased at higher temperatures, whereas negative correlation may stem from increased strength of water stratification at higher temperatures impairing nutrient supply to the photic zone since their vertical mixing is reduced in the productive layer of ocean (Brander, 2007; Arrigo et al., 2008; Boyce et al., 2010). The latter scenario seems to be pronounced in the Tasman Bay where a strong negative correlation between the two variables was observed, but possibly the former scenario in the Hauraki Gulf, thus likely reflecting different effects of SST on fish growth between the two New Zealand sites. Globally, NPP appears to have declining propensities since the commencement of oceanographic measurements in the late 1800s (Boyce, 2010), even though some contrasting reports challenged this generalisation (see Chavez et al., 2011). Nevertheless, it can be expected, at least for low and mid latitude regions, a continuing decline in annual NPP plausibly causing a drop in fish biomass production (Brander, 2007; Blanchard et al., 2012). Therefore, due to complexity of SST/NPP

relationship, and possibility of further global decline in food availability, even though the current study did not specifically aim to establish predictability of 1+ recruitment and YCS strength, it could be suggested that inclusion of NPP on the top of the SST data in efforts for estimating 1+ YCS may be beneficial.

In addition, the growth performance of wild snapper at age 1 also seems to be related and could possibly be a determining factor for the level of performance in second year, similarly as 0+ YCS is likely linked to 1+ YCS (Francis, 1993, 1995). This could be suggested based on a relatively strong correlation (i.e. R = 0.71) observed between fish size at age 1 and age 2. Yet, this was not statistically significant even though the trend appeared obvious. The trend seemed to be lost between second and third year, when snapper began gonadal development and may shift from estuarine to other/deeper habitats (Persons et al., 2014), which also coincides with the ontogenetic sift in dietary patterns (Usmar, 2012).

6.4.3 Are snapper food limited in Tasman Bay, New Zealand?

Among three sources of growth data for juvenile snapper from Tasman Bay (published by Drummond and Kirk, 1986; wild juvenile snapper survey and experimental data from this study) Drummond and Kirk (1986) data demonstrated the greatest length growth at all comparable ages (1–3). However, it is less likely that juvenile snapper from 1980s grew better than experimental fish in the current study kept at unrestricted food regime containing ~50% crude protein that enabled optimum to maximum weight gain (Booth et al., 2008). The bias may come from the fact that measurements were carried out on the fishing vessels, which may compromise accuracy, and more importantly their ageing methodology was not specified nor described. Therefore, the data reported in Drummond and Kirk (1986) could only be regarded as an interesting side note rather than true comparison. However, if sea-water temperature in the 1980s had been lower than present, this may have had a positive effect on the fish, in which case data from Drummond and Kirk (1986) would have been factual. Nevertheless, a much better insight into the question about food limitation for snapper growth in the wild could be derived from a comparison between experimental and wild surveyed data. At age 1 there were no differences between the two cohorts. For the first year, before they entered the unlimited food trials, experimental fish were kept on a maintenance ration, which may be the reason why wild and cultured fish achieved statistically the same growth in length. But since at age 2 experimental fish were significantly larger, had better condition and MLR that indicated overall positive allometric growth, while wild fish demonstrated isometric growth, it seems plausible to suggest that food was the factor that limit the maximum growth that juvenile snapper could achieve in Tasman Bay. Similarly, Arnason et al., (2009) and Guderley et al. (1996) found that Atlantic cod fed

to satiation exhibited positive allometric growth and better growth rates compared with wild cod and the difference was attributed to commonly observed food limitation in the natural environments (Bjornsson, 1999; Bjornsson et al., 2001).

However, investigation into seasonal feeding habits and ontogenetic diet shifts in snapper provided by Usmar (2012) brings a slight twist to the food limitation suggestion. Based on levels of stomach fullness, Usmar (2012) found that irrespective of age snapper from Hauraki Gulf fed all year round. This was also noted for wild *S. aurata* where feeding did not cease through winter but was only reduced (Grigorakis et al., 2002). Usmar (2012) further observed that the lowest level of stomach fullness was in September. This may be explained by the fact that the sea-water temperature was slightly higher in September than in August (see Majed et al., 2002a, 2002b) while the feed intake may have not differed between the 2 months and thus increased temperature in September may have been responsible for increased rate of digestion and stomach evacuation. Furthermore, snapper is a highly adaptable opportunistic predator known to be able to seasonally switch to available prey (Colman, 1972; Russell, 1983; Usmar, 2012). Fish with such an adaptation, according to Weatherly (1963) and Godfriaux (1970), are unlikely to experience any marked interspecific competition or food deficiency in the wild. Bringing it all together, a more plausible suggestion for snapper constraints to manifest their full growth potential could be quality of food items rather than their scarcity, or perhaps a combination of both. This scenario could be viable at least for the Hauraki Gulf population, and testing this hypothesis for the Tasman Bay juvenile snapper, similar work to Usmar (2012) with all year around levels of stomach fullness monitoring would be required.

6.4.4 Are YEM food limited in Tasman Bay, New Zealand?

Curtis and Shima (2005) have provided a report about YEM growth in New Zealand waters that formed a basis for the present study to address the question “is food a growth limiting factor for YEM in the wild?” The findings of the comparison suggest that this may be the case since the experimental fish were over 20% larger after a year of growth than what was estimated for the wild population. However, there are arguments from other studies on Mugilids that may point towards the same suggestion as elaborated for snapper. Cardona (1999) and (2001) were investigating seasonal changes in the food availability and its quality in relation to growth performance of five mugilids from the Mediterranean Sea. It has been suggested that food limitation in Mediterranean coastal lagoons is expected during the warm season rather than in winter (Cardona, 1999; Little, 2000), owing to the seasonal dynamics of detritus turnover, since grey mullet are considered to be predominantly detritivorous (Chubb et al., 1981; Cardona, 2001). Cardona (1999, 2001) found empty stomachs in winter fish, suggesting that even when there was food available (albight with lower nutritional values)

fish appeared to starve. In spring food was at maximum annual levels that dropped in quality and quantity in summer, but remained not limiting. This led the authors to suggest that grey mullet overall were not food limited in the studied habitat (Cordona, 2001). Furthermore, it was speculated that better quality of food supply in summer could possibly augment mullet growth (Cardona, 1999). On the other hand, YEM have been described as omnivorous (Wallace, 1976; McDowall, 1978; Edgar and Shaw, 1995); however, detritus was a commonly found item on the YEM's menu making up to 10 % of their choice (Webb, 1973; Taylor and Paul, 1998). The present study data demonstrated that YEM feed and grow through all seasons, and if the dynamics of recirculation of detritus is similar in Tasman Bay to the Mediterranean lagoons, again it can be suggested that quantity of available food for YEM may not be in short supply but rather its quality may determine the rate of growth in the wild.

In addition, there is another factor that may play a significant role in YEM growth performance. An insight from Curtis and Shima (2005) and several Australian publications (i.e. Harris, 1968; Chubb et al., 1981) indicated the importance of latitudinal gradient on growth of YEM where poleward direction was associated with decrease in growth rates. This may be related to the fact that latitudes are in general negatively correlated with SST where lower latitudes may be associated with higher temperatures causing an increase in metabolic rates and feeding activities (Claireaux and Lagardere, 1999; Sunuma et al., 2007; Arula et al., 2012) and in some instances growth (Rowinski et al., 2015). Besides, species richness has also been found to decrease with latitude (Veron and Minchin, 1992), which could also be an important dietary factor for omnivorous fish such as YEM. The latitudinal gradient does not affect in the same way snapper growth performance since the Tasman Bay population grow faster but it is located at higher latitudes (-41 °S) than Hauraki Gulf snapper (-36 °S).

6.4.5 Did snapper and YEM achieve maximum possible growth rates in the growth experiment?

Experimental GR maximised by the food-unlimited regime assisted in addressing the question about food limitation in the wild. However, feeding fish in excess may not automatically mean that maximum possible GR will be achieved. Across publications, for snapper to achieve the maximum mass gain, it is recommended to use feed with ~50% crude protein. Booth et al., (2008) recommended 52% crude protein for snapper, and for *S. aurata* (Mediterranean region) and *Pagrus major* (Japan) maximal growth results are accomplished on a diet containing 45–50% protein (Foscarini, 1988; Grigorakis et al., 2002). Similarly, in this study, depending on pellet size, the proportion of crude protein in feed ranged between 45 and 50%. The same feeding regime practiced for snapper was also employed for YEM, since there was no published guideline to be adopted. However, there is another aspect of a feeding procedure, namely feeding frequency, that can affect feed conversion ratio and GR

(Jobling, 1986). It is assumed that the amount of food taken at a subsequent meal is equal to the amount of food previously digested (Huebner and Langton, 1982). If the interval between feeding does not allow for a sufficient time for adequate digestion, a subsequent amount of food consumed may cause a phenomenon called gastric overload, which is understood to reduce absorptive efficiency (Jobling, 1986). This was a likely scenario when Nile tilapia, *Oreochromis niloticus*, were fed to satiation with three and five meals a day, since similar amount of feed was consumed under any of the experimental regimes with the results showing better growth performance and feed conversion in the three meals a day scenario (Riche et al., 2004). Booth et al. (2008), however, did not find such a response when conducting feeding trials on *C. auratus* despite observing the highest harvest mass in a fish group fed two times a day compared with more frequent approaches (i.e. 4, 6 or 8 feedings a day). According to that, it is plausible that snapper in the present study fed three to four times a day did not exhibit gastric overload leading to reduced digestive efficiency and consequently experiencing impaired growth performance. To come to the same conclusion was not possible in the case of YEM, as no adequate study was conducted to assist in the assessment. However, YEM are omnivore/detrivore that mostly feed on low-caloric food items (Webb, 1973; Taylor and Paul, 1998) and they are often seen in the natural environment continuously foraging/feeding (personal observation). Therefore, it could be suspected that gastric overload also did not take place in YEM; however, this notion should be experimentally verified. Taking all of this in consideration, it could be suggested that both test species did achieve their maximum growth performance or close to it and that the difference observed with wild cohorts clearly demonstrates that food was the major factor contributing, nonetheless not necessarily due to its lack in the wild but more likely because of seasonal reduction in nutritional values. However, this assumption would also require experimental testing.

6.4.6 Concluding remarks

When it comes to growth of ectotherms, including fish, food is regarded as a main driving force, whereas temperature is a rate controlling factor (Brett and Groves, 1979). As a driving force food resources in natural environments is the key variable restricting maximum level of growth that would be possible according to the capacity that fish have innate in their genetic makeup. If snapper and YEM sampled during the 2015 PFR survey and by Curtis and Shima (2005) respectively well represent the juvenile snapper and YEM population in the designated areas, based on the present research data it can be suggested that food may be limiting for the two test species to express their full growth potential. However, a simple *yes* as an answer to the question may not be adequate enough. Although at some periods food may be less available and certain levels of intra and inter-specific competition could take place, it is also plausible, as elaborated earlier, that both fish have access to food all year round. However, its quality or nutritional value may differ between seasons. This change in annual

dietary levels is therefore more likely responsible for the growth differences observed between cultured and wild fish in the present study.

CHAPTER 7

General discussion

7.1 Two different strategies for survival, fitness and growth: snapper vs. YEM

The choice of model species for this thesis included two somewhat contrasting finfish: snapper that demonstrated a typical temperate growth strategy with pronounced seasonal growth and YEM that, when adequately fed, would grow continuously, resisting the seasonal environmental forces that in the majority of temperate species shape an annual growth trajectory. When both fish, of similar age, entered the annual feeding trials and were kept on an unrestricted diet, in terms of final growth increments, YEM's growth strategy appeared to be superior to snapper's. However, a closer inspection of growth data and associated morphometric indices showed that this may not be necessarily so. Average annual mass increment for snapper was 150.8 g, while for YEM it was 184.8 g, which can be translated into annual % mass increase of 426.0 and 580.6% for snapper and YEM respectively. On the other hand, average annual length growth was 87.4 and 105.1 mm, or as an annual % length increase, 67.3 and 74.3% for snapper and YEM respectively. Therefore, in terms of relative annual length increase results were not markedly different, essentially just reflecting differences in body shapes characterising specific anatomical and locomotory traits of these two fish. But for the mass increase, YEM did appear to perform better. However, when morphometric indices are introduced, particularly visceral lipid index (VLI), it becomes obvious that ~15% of YEM mass belonged to abdominal fat deposits compared to only ~1% for snapper at the end of the growth experiment, indicating much smaller differences in the final somatic growth of the test species. In addition, when gutted mass rather than absolute mass was considered, both species gained on an annual basis a similar amount of mass (~133 g $year^{-1}$) clearly showing that no strategy was superior to another but simply different.

7.1.1 Snapper growth strategy

7.1.1.1 Typical seasonal growth of temperate fish – seasonal compensatory growth phenomena

Compensatory growth is an ability of an organism to accelerate growth following intervals of slow growth caused by reduced food supply or temperature (Jobling, 1994; Schultz et al., 2002). The definition does not necessarily refer to occasional environmental occurrences when either or both (food availability and temperature) are not suitable for continuous growth, but this may point towards recurrent seasonal events when these variables oscillate regularly and predictably, which is especially emphasised in high latitudinal regions. More poleward fish species are acclimated to a shorter growth season (Jobling, 1997). According to this it could be concluded that fish from higher latitudes are disadvantaged from an ecological point of view since a shorter annual growth season may mean smaller annual growth increments in both length and mass than what would be possible at lower latitudes. Lower latitude fish potentially grow faster, likely decreasing mortality rates due to predation as well as increasing reproductive output compared to high latitude populations (Clarke, 1993). In many instances this predictable pattern has been observed; however, the growth differences between populations occupying lower or higher latitude are often much smaller than what would be expected based on temperature and duration of the growth season differences (Conover, 1990, 1992). There have been proposed three, not mutually exclusive, scenarios to accommodate this phenomenon – first that in high latitudes mortality is strongly size-selective and only the largest, fast growing fish survive; second – at low latitudes there might be much higher intra and inter-specific competition resulting in fish performing below their full growth potential; and third, that certain compensatory mechanisms are in place to offset the adverse influence of a shorter growing season and thermal constraints experienced at high latitudes (Jobling, 2008). Two models have been proposed to explain the presence of compensatory growth at higher latitudes. The *thermal adaptation* model suggests that temperature for optimum growth is negatively correlated with latitude, or in other words temperature for optimal growth matches with annual temperature average at different latitudes (Jobling, 1997; 2008; Craig, 2000). This suggestion has been mainly refuted since in many cases the optimal temperature for growth has been found to be similar between low and high latitudinal populations (Conover and Present, 1990; McGeer et al., 1991; Kaya et al., 1992). An even stronger argument against it comes from New Zealand waters where snapper populations from Tasman Bay (higher latitude; this study) were found to grow overall faster than populations from the Hauraki Gulf (lower latitude; Francis, 1994a). Second, the *countergradient* model proposes that populations from high latitudinal environments grow faster to primarily compensate for the shorter growing season rather than for decreased temperatures compared to low latitudes (Jobling, 1997; Craig, 2000; Larsson, 2002), where regions with the most hostile traits are associated with populations possessing

the fastest growth genotypes (Conover and Schultz 1995). Schultz et al. (2002), working on three populations of juvenile Atlantic silverside (*Menidia menidia*) inhabiting different latitudes, demonstrated that the growth strategy of higher latitude fish was characterised with a faster recovery after winter growth cessation/depression joining other specialised adaptations that have differentiated along an environmental gradient in seasonality, such as greater tolerance for cold (Schultz et al., 1998), greater reproductive rates (Klahre, 1998) and greater energy storage (Schultz and Conover, 1997).

This type of rapidly accelerated growth once conditions improve as seasons change does not necessarily mean that all tissues exhibit growth at the same rate. Compared with fish that exhibited routine growth (constantly fed), fish displaying compensatory growth may have lower relative white muscle (WM) mass but at the same time relative intestinal (Bélanger et al., 2002), heart and liver (Ali et al., 2003) mass may be increased. Bélanger et al., (2002) found that increased relative intestinal mass could be coupled with increased enzyme activity involved in aerobic energy generation (i.e. citrate synthase, CS), indicating that size and aerobic/mitochondrial activity of the digestive tract may be linked with compensatory growth capacity (Bélanger et al., 2002). Even though Bélanger et al. (2002) findings stemmed from work where seasonal compensatory growth was not directly investigated, the entero-somatic index (ESI, relative intestinal mass) as well as activity of intestinal CS of snapper in the present study followed this notion when growth was reinstated following winter. This observation further supports the suggestion that snapper growth is a type of large scale annual compensatory growth.

The seasonal growth pattern as observed for fish inhabiting temperate and polar environments is often associated with the temporal fluctuation in availability of food resources, yet even if food as a growth factor is not limited, based on temperature reduction only, it would be expected that growth would be impaired (Jobling, 2008), as was the case during the snapper growth trials. Although temperature is an indisputably crucially important driver, seasonal growth cycling cannot be solely attributed to temperature effects but it appears that high latitude fish follow a seasonal cycle of photoperiod (Jobling, 1994; Boeuf and Le Bail, 1999), which acts as a *zeitgeber* (German for 'time givers') to entrain an endogenous growth rhythm with prevailing environmental conditions (Jobling, 2008).

7.1.1.1.1 How does this work? – snapper seasonal growth pattern and endogenous clock

Every organism is subject to environmental forces. Some may appear haphazardly and, by definition are unpredictable in nature (e.g. encountering predator, cloud shading, infection), while others are associated with events such as phases of daylight, tides, lunar cycle or seasons, thus they

can be predicted if a mechanism to detect specific environmental change was in place (Madrid et al., 2001). Such an ability, where an organism can "foresee" immediate or more distant future circumstances and can be adequately prepared to maximise benefits of life supporting conditions as well as minimise potential hazards associated with those events, is an important survival strategy. Accordingly, an array of diverse timekeeping systems to fine-tune physiology and behaviour to environmental cycles have been recognised (Madrid et al., 2001).

Living organisms determine time by combined inputs from two sources: by monitoring the exogenous periodicity of the environment and by acting upon a guidance of internal processes for time measurement, such as endogenous circadian or circannual clocks (Aschoff, 1981; Pittendrigh, 1981). Relying only on external time cues to shape animals' behaviour would be maladaptive as they may rapidly lose the capacity to predict important events if such cues were withdrawn from their immediate environment (Sánchez-Vázquez and Madrid, 2001). This scenario is likely not the case as many studies showed the opposite, therefore it has been suggested that a hypothesis founded exclusively on external cueing can be discounted (Sánchez-Vázquez and Madrid, 2001). For example, Sæther et al. (1996) demonstrated for Arctic charr (*Salvelinus alpinus*) that seasonal cycles in feeding behaviour and growth performance were preserved even when fish were kept under constant temperature (4°C) and photoperiod (12 hours light, 12 hours dark) showing that circannual rhythms continued in the absence of seasonal changes in these environmental cues. Maintenance of circannual clocks requires a defined entraining process synchronised by gradual changes in both photoperiod and temperature and probably other unidentified external cues (Madrid et al., 2001). Therefore, seasonal changes in proximate factors (daylight, temperature) do not actively induce changes in behaviour and physiology, but rather can be considered *zeitgebers* that entrain endogenous circannual rhythms (Gwinner, 1986).

In vertebrates one of the most prominent systems often found associated with endogenous circadian/circannual clocks entraining daily and annual physiological rhythms is the hormone melatonin (Falcón et al., 2010). It is produced by the pineal gland and the retinal cells and the duration of the neurohormonal message involved in production and function of the hormone matches periods of darkness, therefore it is regarded as an internal *zeitgeber* of the organism (Falcon et al., 1992; Zachmann et al., 1992). Since it is exclusively synthesised at night, following pineal and retinal cell depolarisation (Falcón, 1999), it can supply an organism with accurate timing of daily and calendar time based on photoperiodic signals (Randall et al., 1995; Chowdhury et al., 2008).

Endogenous circannual clocks, rather than being directly affected by low winter temperatures, can potentially explain the fact that snapper in this study, independent of unlimited food supplied throughout the course of the growth study, ate minimally in winter, and growth was markedly reduced or even negative. Snapper is a highly visual forager (Robinson et al., 2011, 2013, 2017) that also

changes daily feeding periodicity on a seasonal basis – being predominantly nocturnal in winter, but diurnal and nocturnal in summer and spring/autumn respectively (Coubrough et al., 2004). Therefore, it can be concluded that visual predators, such as snapper, rely heavily on their vision to communicate with the environment on a daily basis as well as to entrain their inner biological clock to measure and understand time.

In relation to predictable seasonal periodicity and associated internal clock, fish often develop an anticipatory response in both feeding behaviour and physiological/biochemical adjustments, so that physiological processes can be initiated prior to an external periodic event (Madrid et al., 2001). This type of response was also observed in snapper in this study. Snapper are capable of utilising photic and likely thermal signals from the environment and carry out specific biochemical preparation of tissues important for intense (compensatory) growth before a growth season commences in spring as well for the growth break in the later season. The example of this was found in aerobic (CCO/CS) activity in WM and partially in the intestine (Chapter 5). Furthermore, one of the most obvious themes that characterised the annual snapper growth pattern – maximising energy stores and condition in autumn, as seen in both morphometrical data (Chapter 2), and biochemical data (Chapter 5), as a preparation for winter was also likely governed by the endogenous circannual clock system.

7.1.1.2 Snapper seasonal growth pattern and temperature

Snapper annual feeding and growth strategy can also be explored and explained in terms of temperature effects alone. It has been well established that snapper growth is strictly seasonal and that their growth rates (GR) would consistently correlate positively with ambient sea-water temperature either when fish growth was measured in the wild (Francis, 1994a) or in an enclosed aquaculture rearing facility (this study). In addition, Coubrough et al. (2004) pointed out that snapper feeding behaviour was affected by temperature and that the relationship was not linear but rather step-wise, whereby at temperatures below 11°C feeding was minimal, between 11 and 18°C was intermediate and above 18°C feeding was markedly increased. Another investigation, carried out by Jerrett et al. (2002), aimed to determined snapper post-mortem metabolic measurements in relation to post-mortem storage temperature. They indicated that a 16–18 °C temperature level was particularly important and it was described as a metabolic shift between a winter and summer mode which was also supported by behavioural differences noted in captive fish. In the present study, an interesting observation was made when internal organ mass and associated morphometrics were correlated with total body mass throughout 12 experimental months (Fig. 2.36). Except for heart mass, all other measurements demonstrated the highest and the most uniform levels of correlation with body mass at temperatures that matched with 16–18°C.

These high and uniform correlations may mean that all parts (organs and tissues) that constitute an organism are the most synchronised and contribute equally to snapper wellbeing/fitness in terms of being prepared for winter as well as catching-up in somatic growth in spring when 16–18°C temperatures are dominant. Could that mean that this temperature range is optimal for overall snapper fitness and/or when snapper operate the most efficiently? According to Jobling (1997, see Chapter 1 for illustration) temperatures where ingestion rate is maximised are above the temperature for optimal growth, which in turn is above the temperature that sustains maximal feed conversion efficiency (FCR). Furthermore, Handeland et al. (2008) stated, since optimal temperature for growth is often higher than fish usually experience in the wild (which is the case for snapper in the present study, see Chapter 2), that the most important physiological feature for maximising individual fitness is to maximise food utilisation rather than growth. According to what has been elaborated here, it is plausible to suggest that the 16–18°C temperature range is an optimal thermal condition to support snapper fitness and wellbeing. However, it would be necessary to conduct investigations to determine if ~17°C is when FCR is indeed maximised. In addition, it would be imperative to know how that might vary amongst areas where snapper fisheries in New Zealand waters are economically exploited, as this information may be applicable/vital for future investigation into possibilities of traditional snapper aquaculture, sea farming or free ranging.

The final link to connect the circle of arguments about snapper growth strategy can be found in snapper lifestyle. Snapper are shoaling finfish inhabiting harbours, estuaries, reefs and open coastal areas (Paulin, 1990) inhabiting depths ranging from 10 to 200 m (depending on age and season, as in winter they tend to move into the deeper zone; Francis, 1995). They are generally demersal, therefore not usually exposed to rapid temperature changes associated with shallower or surface waters. They are considered as long-lived species (over 60 years of age, Parsons et al., 2014) and mature between the ages 2+ and 5+, depending how quickly they reach a maturation size of ~25 cm (Francis and Pankhurst, 1988). Taking these life history traits into consideration it becomes apparent that year-round maintenance of growth is not essential for snapper and that feeding can be suppressed when the surrounding temperature is not supportive of energetically costly foraging activities (Coubrough et al., 2004). In addition, as described in Chapter 6, snapper do feed all year around, but at an evidently reduced rate during winter, likely just to maintain a balanced utilisation of energy reserves that are required to last through the cold season, and the only restriction for the faster growth in the wild may not be food shortage but rather its quality.

7.1.2 YEM growth strategy

7.1.2.1 Similarities with snapper

Both YEM and snapper are temperate teleosts, hence it is expected to find some similarities in both biological and ecological traits to link the two species. Generally, they are both social, particularly as subadults; however, this trait seems to be more important for YEM as it lasts throughout their lifespan, which may not be the case for snapper (Parsons et al., 2014). Further, they are both visual foragers and often as juveniles can be found inhabiting the same coastal environments.

Among similarities found in the present study it is worth mentioning that both fish responded equally in terms of change in hepato-somatic index (HSI), as a function of seasonal temperature change. HSI in both species was the highest in winter, when growth slightly slowed in YEM but stopped, reversed or was minimal in snapper. This scenario was also observed for wild snapper in Francis (1997), where it was proposed that energy reserves were exhausted during intense growth in spring/summer and replenished when growth was minimal in winter instigating HSI increase. This suggestion could not be supported by current findings where it was observed that liver glycogen depleted through the winter in both test species rather than restored (Chapter 5). The negative relationship between HSI and ambient temperature phenomenon has not been frequently reported; however, it has been observed in a few other species, such as gilthead sea bream *Sparus aurata* (Grigorakis et al., 2002); rainbow trout *Oncorhynchus mykiss* (Hilton, 1982) and roach *Rutilus rutilus* (van Dijk et al., 2005). Grigorakis et al., (2002) argue that the reason for this apparent anomaly may be due to liver metabolic malfunctioning caused by adverse effects of low temperatures as a proposed mechanism for high winter HSI values also found in rainbow trout by Hilton (1982). This could possibly be the case for snapper, since the appearance of *green* liver in winter was common indicating some possibilities of pathophysiological processes in the organ. The *green* liver in cultured red sea bream *Pagrus major* has been related to low hepatic taurine levels accompanied by an accumulation of biliverdin (a pigment derived from haemoglobin degradation in the liver) (Goto et al., 2001), and this state in snapper could be related to the occurrences of the winter syndrome as mentioned in Chapter 2. However, in overwintering YEM liver, no organoleptic pathological signs could be noted in the present study, therefore it appears that there is not an adequate explanation for the annual HSI dynamics as observed in aforementioned studies including the present one.

Another similarity between test species was observed in the direction of correlation of activities of certain enzymes and their scaling with growth parameters (mass/length) and age (ontogenetic effects) in examined tissues (white and red muscle, RM) and organs (intestine and liver) (Appendix 2). Enzyme activity dependence upon fish size (mass or length) was observed in many cases but often

a stronger relationship was noted when activities were regressed against fish age indicating primarily ontogenetic effects on activities of energy pathways represented with these enzymes (Appendix 2). Cytochrome *c* oxidase (CCO) in red muscle (RM) did not change for both fish as a function of either parameters, likely indicating that the capacity of the electron transport chain for ATP synthesis in RM was sufficient to meet the demand for energy generation when its function was maintained per gram of wet tissue irrespective of fish age or size (Blier and Lemiex, 2001). 3-hydroxyacyl-CoA dehydrogenase (HOAD) activity tended to decrease with age/size in WM of both species but increase in RM, demonstrating a reduced and enhanced reliance on lipid oxidation for energy production as fish age/growth in WM and RM respectively. However, the most similar patterns between the two model species in terms of enzyme activity and fish age/size was observed in activity of glycolytic enzymes pyruvate kinase (PK) and lactate dehydrogenase (LDH). PK and LDH activity increased in WM of both fish with size/age and decreased in RM, clearly illustrating the importance of anaerobic glycolysis for burst swimming performed by WM as fish grow and increase in size (predominantly in mass). Analogous scaling of activity of glycolytic enzymes has been observed also for wild snapper (Majed et al., 2002b). Since glycolytic enzymes showed the most uniform response as a function of fish size in both species, they likely demonstrated an equal purpose for such a response. Growth in fish is largely attributed to an increase in muscle mass; however, this fact is associated with a hydrodynamic disadvantage, which is especially significant in an aqueous medium where bigger mass means greater drag, which in turn may cause reduction in accelerating power (Mommsen, 2001). Transient propulsion is a vital survival strategy since rapid acceleration and quickly executed turns are equally required to catch and escape (Weihs and Webb, 1983). The main force acting on a moving body in fluid, such as water, is hydrodynamic drag that acts against the direction of the motion and it tends to increase with speed. To overcome it in order to maintain the ability for burst swimming independently of their size (i.e. that fish of different lengths can attain the same relative speeds, as expressed in body length per second, during peak burst swimming) fish need glycolytic metabolic power to increase/scale substantially faster than muscle mass as fish grow (Somero et al., 1980). Somero et al. (1980) found a marked increase in LDH and PK activities in white muscle of 13 teleosts where, in general, the scaling of glycolytic capacities matched with power required for burst acceleration, based on computed drag scaling with body size and relative swimming velocity. Therefore, strong upward scaling of glycolytic activity with snapper and YEM size can be plausibly explained with the scenario described in Somero et al. (1980). Further, they suggested that an ecological relevance for the preservation of burst-swimming capacity relative to body length is based on the relation between prey/predator size and the distance they can be detected, which increases with size.

7.1.2.2 Differences with snapper

Many fish exhibit seasonal growth variability. This trait is not exclusive for temperate and polar regions, since even in the tropics seasonality may affect fish growth performance (Jobling, 2008). Nevertheless, if provided with adequate quality and quantity of food, YEM from Tasman Bay, New Zealand, exhibit almost linear annual growth, as this study demonstrated. In addition, YEM has been reported to grow linearly for the majority and possibly its entire lifespan in natural habitats around New Zealand (Curtis and Shima, 2005), indicating a rare occurrence of non-asymptotic growth in fish. However, this suggestion may be controversial since a comparable study conducted in South Australia (see Harris, 1968) reported asymptotic growth of YEM. Nevertheless, researchers would unanimously agree that the total lifespan of YEM, independent of locality studied, would not generally exceed 7 years (Thomson, 1957; Taylor and Paul, 1998). Thus YEM, as was also stated for the striped mullet (*Mugil platanus*, ~10 years lifespan) is considered shorth-lived even within the family of Mugilids (Paul, 2000; Castro et al., 2009). Relatively low longevity of YEM, together with other life history traits (e.g. early maturation, euryhaline and eurythermal adaptations, high GR and constant growth), portray YEM as an *r* selected species, which is in opposition to long-lived snapper (Paulin, 1990). Reasons for the difference in longevity between the two test species may be related to differences in their metabolic rates (Chapter 3), since animals with faster metabolism were linked to shorter lives (Ku et al., 1993). Besides, several authors pointed out that along a slow–fast metabolic continuum, animals that are placed towards the fast end have generally more rapid growth, higher metabolism, lower investment in maintenance and lower longevity (Williams et al. 2010; Wiersma et al., 2012). YEM have been found to have overall lower maintenance requirements (resting/routine metabolic rates, RMR) than snapper but a markedly (up to two times) higher capacity for maximum oxygen utilisation (maximum metabolic rate, MMR), which produces over two times higher aerobic metabolic scope that YEM require for intense foraging, continuous feeding and growth. But the fact that YEM are very active and have high aerobic capacity cannot explain their low longevity. The answer may come from the biochemical work carried out in the current study.

Majed et al. (2002b) found for snapper that CS activity in white muscle declined with fish size, which was also the case for the snapper in the present study, suggesting reduction in energy generation as fish grow, since smaller fish may have higher metabolic capacity and faster GR than larger fish. This was also true for CCO activity in snapper WM. However ontogenetic/allometric responses of CS and CCO activity in YEM WM was contrary to those observations for snapper (Majed et al.,2002b, present study), rainbow trout (Kiessling et al., 1991), sand bass, kelp bass (*Paralabrax clathratus*), slippery dick (*Halichoeres bivittatus*) and many others (Somero et al., 1980). Therefore, instead of decreasing aerobic activity in WM as they got larger, YEM tended to increase it. This suggests that the capacity to grow in younger/smaller YEM is not superior to older/larger fish but rather the opposite. Is it possible

that an allometric increase in the aerobic capacity of WM can be linked to a shorter lifespan of YEM? As depicted in Chapter 5, activities of both aerobic mitochondrial enzymes (CS and CCO) in YEM WM strongly positively correlated, it is safe to suggest that the mechanism for the activity increase was accomplished by increasing mitochondrial density. The consequence of having higher numbers of mitochondria in a tissue may be maladaptive as more mitochondria generally mean higher rates of proton leaks and generation of reactive oxygen species (ROS) that are produced during electron transport in mitochondria (Guderley, 2004). On average 1–3% of consumed oxygen by an organism is converted into ROS, which are now known to be used as cell signalling factors and any excess is scavenged by cellular/endogenous antioxidant systems (e.g. superoxide dismutase) and vitamins A, C, and E (Buehler, 2012). The rate of ROS production may exceed the neutralising capacity of the antioxidant system resulting in oxidative stress (e.g. lipid membrane damage, increase in DNA mutations, mitochondrial depletion, cell death) (Buehler, 2012). According to the free radical theory of aging (Harman, 1956), cellular damage instigated by ROS is a main driving force of aging and major determinant of longevity. In recent times it has been demonstrated that ROS do not directly cause a stress response to age-dependent damage but that they rather play a role in mediating the process (Hekimi et al., 2011). Accordingly, it seems obvious that likely increases in mitochondrial abundance and resulting aerobic activity could relate to increased oxidative stress in YEM WM that may at least in part be responsible for the relatively short lifespan of this temperate, coastal species.

When the same approach was used to correlate internal organ mass and associated morphometrics with total body mass (Fig. 2.37), as was carried out for snapper, the highest correlations and overall the most uniform responses were observed at a temperature range of 10–16°C. But, as explained earlier, this response is likely not related to ambient temperature. This range, extending from mid-winter (July) till late spring (October), corresponds with a period when lipid deposition was (almost) linearly increasing through winter until the optimal levels of ~15% BM was reached in October (Chapter 2). If the scenario about temperatures and efficiency, as explained for snapper, can be applied to YEM, during this period YEM was plausibly operating most efficiently. At least it appears that during the time of energy rebuild for both species correlations depicted in Fig. 2.36 and 2.37 were highest and the most similar possibly bringing all physiological systems to the most synergistic and synchronised operation. However, this may be related to the unlimited food regime fish were submitted to in the feeding trials. Wheter the same response as a function of body mass would be found if test species were kept on maintenance ration is yet to be confirmed.

7.2 Metabolic rates, growth and effects of temperature

Three hypotheses have been proposed aiming to explain the association between metabolic rate and growth. The increased intake hypothesis is based on the possibility that a higher resting metabolism is related to maintenance of a system that promotes higher MMR (e.g. larger internal organs, Chappell et al., 2007), which can result in greater aerobic scope (AS) and rates of nutrient processing and assimilation (Biro and Stamps, 2010). In this case resting metabolism would have a positive association with growth and fitness (Burton et al., 2011). Second, the compensation hypothesis would predict the opposite. Lower maintenance costs may allow a greater percentage of resources to be directed towards non-essential activities such as somatic and/or reproductive growth (Wieser, 1994). Accordingly, RMR would be negatively correlated with growth (Burton et al., 2011). And lastly, the context-dependent hypothesis predicts that the way resting metabolism relates to GR and fitness will depend on specific conditions animals may be exposed to in different environments (Burton et al., 2011). General observation suggests that when there is an abundant food supply (e.g. laboratory conditions with unrestricted diet) that the first (increased intake) hypothesis may be prevalent, but when food supply is reduced or access to it is less predictable (e.g. the natural conditions) the relationship becomes negative (Reid et al., 2011, 2012; Auer et al., 2015) or it is non-existent (Finstad et al., 2007; Norin and Malte, 2011).

Studies seeking to explain an RMR/GR relationship do not necessarily incorporate temperature effects. Nevertheless, in the present study metabolic rates were determined at different temperatures (e.g. 13, 17 and 21°C) and when these data were correlated with GR that fish fed *ad libitum* accomplished at corresponding temperatures (Appendix 3C) it was obvious that the way metabolic rates and GR related differed between the two test species. Snapper demonstrated typical responses in support of the increased intake hypothesis, where both RMR and MMR appeared positively correlated with both specific mass GR (SMGR) and specific length GR (SLGR), which was further confirmed when data from Coxon (2014) were incorporated, increasing statistical power and thus demonstrating a significant level of relationships (Appendix 3C). By contrast, YEM correlation with SMGR was very weak and likely not present; however, both metabolic parameters, particularly RMR, exhibited a much stronger link to SLGR. According to this, it seems obvious that a species-specific attribute should be taken into consideration when proposing a hypothetical explanation of association of metabolic rates with fish growth performance and fitness.

A study that incorporated temperature effects in relating growth, aerobic scope and temperature (Khan et al., 2014) hypothesised, while investigating optimal temperature for growth of hapuku (*Polyprion oxygeneios*), that maximal GR and feed efficiency would be found at temperatures where

AS was equally maximised. They specified that their results were aligned with the oxygen and capacity limited thermal tolerance (OCLTT) hypothesis, since the greatest growth performance occurred at temperatures where AS was at its peak, as has been found for some other teleosts such as Atlantic cod, *Gadus morhua* (Claireaux et al., 2000), and sockeye salmon, *Oncorhynchus nerka* (Brett, 1976). The present study, in addition to what was already stated about OCLTT in chapter 3, did not find support in respect of this relationship. Snapper AS for the three experimental temperatures, appeared to show a positive correlation with SMGR/SLGR data (Appendix 3C). When the number of data pairs were increased with AS data from three additional temperatures from Coxon (2014), coefficient of determination (R^2) explaining the data dropped from ~0.84 for the three data pairs (this study only) to ~0.16 for six data pairs (combined data from this study and Coxon, 2014), none yielding significant results (Appendix 3C). In the case of YEM, three data pairs acquired in the present study produced a very low value of R^2 indicating the lack of relationship between AS and GR for the experimental array of temperatures (Appendix 4). Overall the present work cannot support the underlining idea that at the temperature when AS is maximised all fitness related functions (e.g. growth, reproduction) will also be maximised, as the core idea governing OCLTT. According to a set of authors opposing the eco-physiological relevance of the paradigm, the hypothesis is progressively being redefined, which even further impairs its predictive power and testability (Jutfelt et al., 2018). Despite increasing evidence against the predictability and testability of OCLTT, debate about its eco-physiological relevance continues (see Pörtner et al., 2017; Jutfelt et al., 2018).

7.3 SDA and growth

According to several studies the digestive capacity of certain fish may be more closely related to their maximum attainable GR than levels of available food in the wild (Kvist and Lindstrom, 2000; Speakman and Krol, 2006). Since an increase in metabolic activity, following meal consumption, is a metabolic response that encompasses all digestive activities, it has been suggested that a postprandial increase in aerobic respiration may be linked to the potential maximum GR (Fu et al., 2008). In addition, it has been proposed by Jobling (1981b) initially, and subsequently demonstrated by other authors (e.g. Brown and Cameron, 1991; Whiteley et al., 2001; Seth et al., 2010), that ~80% of all energy involved in SDA is related to post-absorptive costs, predominantly protein turnover and growth, thus framing SDA as an inevitable cost of growth. Support for the notion that a larger SDA response could be associated with better growth (Grigoriou and Richardson, 2008; Seth et al., 2010) was found, for example, in fast-growing transgenic *coho* salmon, *Oncorhynchus kisutch*, that exhibited greater SDA metabolic rates than slow-growing wild conspecifics (Leggatt et al., 2003). Supporting

data were also obtained from several species of the Cyprinidae family (e.g. the rock carp, *Procypris rabaudi*, Li et al., 2013a and the grass carp *Ctenopharyngodon Idella*, Carter and Brafield, 1992), but not in some others, such as in qingbo carp, *Spinibarbus sinensis* (Li et al., 2013b). In addition, the SDA/GR relationship was not found in sedentary catfish, *Silurus meridionalis*, where the GR and SDA peak did not correlate, likely reflecting the catfish sit-and-wait lifestyle and related energy budgeting strategy (Fu et al., 2008). Khan et al. (2015) found in juvenile hapuku that the greater SDA response was associated with an increase in GR only under certain combinations of rations and temperatures where interaction between the two parameters played an essential role in the strength and existence of the SDA/GR relationship. Taking all this into account it appears that generalisation within the realm of fish is not permissible and that the relationship is not always simple but rather complex.

To propose a clear interpretation about the relationship between SDA response and GR in snapper and YEM was not entirely plausible based on the current data. Insufficient YEM SDA data does not allow speculation about SDA/GR for this species, and constraints for such an interpretation for snapper are found in the fact that growth data were generated with fish on maximum rations (unlimited food supply) likely governed by seasonal change in ambient temperature, while SDA data were based on set rations of 0.5, 1, 2 and 3% BM (body mass) at 17°C for snapper; and at set ration of 0.5% for 13, 17 and 21°C (Chapter 4). To some degree it would be possible to estimate the meal size that fish consumed when fed in excess at any given temperature by utilising a DFC (daily feed consumption) curve described in Chapter 2. However, to further estimate values of SDA parameters, based on the DFC estimated meal size, would likely increase the margin of error as the allometric/scaling relationship is not known between ration size and SDA responses at other temperatures than 17°C (this study). Therefore, extrapolation from the curves explaining SDA parameters data may not be factual. Thus, to examine the relationship, a carefully designed experiment, similar to what was described in Khan et al. (2015), should be carried out. Nevertheless, assuming that a general pattern of SDA parameters, as observed for four rations at 17°C (Chapter 4) would at least preserve the direction of the relationship (i.e. positive/negative) with seasonal temperature change, it can be speculated that snapper would demonstrate a positive relationship between SDA and growth (Appendix 5). This is because SDA parameters when correlated with GR at corresponding temperatures exhibited a direction that promotes growth (e.g. SDA peak and cost increased and SDA duration decreased with SMGR/SLGR, Appendix 5). In addition, a snapper relative, *S. aurata*, has also demonstrated a possibility that higher SDA response at higher temperatures may be related to greater capacity for growth (Requena et al., 1997). Since the history of SDA investigation demonstrated that there was no universal metabolic response in how would fish react to ingested meals and how this may be reflected in growth parameters, the complexity in response may be species and context dependent (Khan et al., 2015).

7.4 Proposed future research

In addition to what has already been proposed for further investigations throughout the discussions, there are a few topics in relation to the objectives of this thesis that may require special attention in the future.

One of the objectives in this work was to determine the benefits of supplementary feeding on growth performance by implementing an unrestricted feeding regime while keeping other environmental variables oscillating in their natural rhythm. Since experimental fish did exhibit enhanced growth compared to wild populations (Chapter 6), it would be sensible to expand this work to a natural habitat as carried out in Bjornsson, (2002) and Bjornsson et al. (2010). This suggestion is more applicable for snapper, as one of the most profitable and iconic of New Zealand's fisheries (Mossman, 2008), whose production boost is required and would be greatly welcomed on both domestic and international markets (Forest and Bird, 2009). Implementing analogous strategies to conditioning (i.e. using the classical Pavlovian conditioning) wild cohorts as per Bjornsson (2011) and Fujiya et al. (1980) and to carry out the supplementary feeding based on low cost trash fish would permit determination of actual levels of test species enhanced growth production. This is because GR of fish kept in tanks at constant density with a lack of environmental enrichment (Brown et al., 2003) may markedly differ from what is possible in the wild. This step should be next in exploring a relatively new approach of fish free ranging due to an array of its potential benefits. The fish free ranging strategy aims to increase annual harvest of valuable ocean proteins while maintaining low production cost, compared with costs associated with traditional fishing practices, as well as minimising effects on the environment (Bjornsson, 2001). The implementation of the free ranging strategy would also be acceptable from an economical point of view for business owners as well as for general consumers since it is expected that less resources would be required for harvesting which may be reflected in lowered retail prices (Bjornsson, 2001). In relation to exploration of snapper growth potential, information about their capacity of compensatory growth is missing which, when adequately utilised, can contribute to lowering production costs (Ali et al., 2003). Hence, this information may have applications in aquaculture/sea-farming conditions where a feeding regime can be artificially manipulated. The idea of snapper farming in New Zealand is not new; however, it has not sufficiently been explored to be a viable option (Scott and Pankhurst, 1992; Booth and Cox, 2003; Parsons et al, 2014). Findings from kin species in respect to capacity for growth compensation are conflicting. *S. aurata*, as a well investigated species from the Mediterranean region that is regularly used for comparison with *C. auratus*, demonstrated lack of growth compensation in length potentially limiting maximum attainable mass increase (Bavčević et al., 2010). However, *Pagrus major* did perform full compensation as reported for fish from the sea farming station of Korean Ocean (Oh et al., 2007). Since

phylogenetic relatives to Australasian snapper did not produce comparable results it is not clear how *C. auratus* would perform when subjected to a growth compensation trial, therefore it would be important also to know this aspect of snapper biology.

Behavioural thermoregulation is an important feature for fish strategy to survive and enhance fitness and utilisation of these traits can greatly complement a growth study such as depicted in this thesis. It has been proposed and demonstrated for some species that fish would select and spend most of their time in water at temperatures that support highest GR, whereby lower temperatures are selected when food is in low supply and opposite when food is abundant (Stuntz and Magnuson, 1976; Mac, 1985; Jobling, 1997). High food supply may be related to selection of higher temperatures as this strategy endorses rapid digestion and nutrient assimilation allowing more frequent feeding that promotes enhanced growth performance (Neverman and Wurtsbaugh, 1994), whereas food deprivation may lead to selection of a colder environment that will support energy conservation as maintenance metabolic rates would drop (Jobling, 1997). Investigating how food deprivation and food provision (i.e. postprandial metabolism) affects the preferred temperature of test species can introduce insights in how to further improve and maximise growth production in aquaculture environments. As demonstrated in Khan et al. (2014) where behavioural thermoregulation was a means to linking growth and FCR with preferable temperature in a novel aquaculture candidate hapuku (*Polyprion oxygeneios*), temperature preferenda can be an effective tool in determining the optimum temperature for growth (Jobling, 1981a). A determination of the optimum temperature was not possible for snapper in the present study and it has not been established in any former works, thus the knowledge about it remains unknown, although the suggestions have been made in this thesis (for details see Chapter 2).

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APPENDIX 1

Use of anaesthesia and ethical approval

Two types of anaesthetics were used during the course of the thesis. A derivative of *p*-aminobenzoic acid, MS-222 (ethyl 3-aminobenzoate methanesulfonate, Sigma-Aldrich) was used at the University. MS-222 is the most widely used anaesthetic for poikilotherms, including fish (Tytler and Hawkins, 1981). Use of another anaesthetic AQUI-S™ (AQUI-S, New Zealand LTD, Lower Hutt, New Zealand) was practiced at the PFR facilities. AQUI-S™ is based on the sedative properties of eugenol from clove oil (Iversen et al., 2003). It is recognised as safe (i.e. food grade) by the American Food and Drug Administration (FDA) (Ross and Ross, 1999).

Stages of anaesthesia in fish (from Schoettger and Julin, 1967):

Stage	Descriptor	Behavioural response
1	Light sedation	Partial loss of reaction to external stimuli.
2	Deep sedation	Partial loss of equilibrium, no reaction to external stimuli.
3a	Total loss of equilibrium	Fish usually turn over but retain swimming ability.
3b	Total loss of equilibrium	Swimming ability stops, but responds to pressure on the caudal peduncle.
4	Anaesthesia	Loss of reflex activity, no reaction to strong external stimuli.
5	Medullary collapse (death)	Respiratory movement ceases (death).

A list of anaesthesia carried out for different procedures throughout the thesis with associated stages in parentheses:

- Transport Snapper from PFR facilities to Christchurch – 5 ppm AQUI-S (stage *1* reached within 15–20 min)
- Taking measurements of snapper at the University before respirometry work – 75mg L^{-1} MS-222 (stage *3a* reached within 5 minutes)
- Capturing YEM in the wild and their transport to the PFR facilities – 10 ppm AQUI-S (stage *2* reached within 15–20 minutes)

- Taking measurements of snapper and YEM at the PFR facilities before respirometry work – 15 ppm AQUI-S (stage *3a* reached within 20 min, temperature dependent)
- Growth tank array experiment – tagging snapper and YEM (25 ppm AQUI-S, stage *4* reached within 30 minutes) and monthly measurements sessions including measurements and scales taken from the wild snapper (20 ppm AQUI-S, stage *3b* within 30 minutes respectively, temperature depended).

The use of anaesthetics at the University and PFR was approved collectively with Ethical applications by the University of Canterbury Animal Ethics Committee. The signed documents were IDAO (Institutional Drug Administration order) 18-14/01 with its Ethical approval 2014/ 18R; and IDAO 29-14/01 with its Ethical approval 2014/29R for the University and PFR work respectively.

APPENDIX 2

Relationship between snapper and YEM age, body length and mass with activities of aerobic and glycolytic enzymes

Introduction

The purpose of measuring enzyme activities (chapter 5) was not to specifically address a question about fish size (mass/length) and age (ontogenetic) effects on their activities in the selected tissues (white and red muscle, intestines and liver). Nevertheless, the opportunity to do so revealed interesting patterns. The most prominent ones were discussed in chapter 7, such as possibilities for YEM's low longevity. Activities of aerobic (mitochondrial) and anaerobic/glycolytic enzymes have been often explained in the light of allometric scaling with an increase in body size or as a function of age (Majed et al., 2002b, Somero et al., 1980; Kiessling et al., 1991). Change in fish size/age is associated with change in specific metabolic requirements which is not necessarily the same across all tissues and organs in the body. Thus, some organs/tissues increase, decrease or do not change energy demand generated from certain metabolic pathways as fish age and/or increase in body size.

Methods

Data used for this appendix were the same as generated in chapter 5.

In terms of analytical/statistical procedures and calculations, all enzyme activity data were analysed with the general linear model (i.e. regression analysis), in the fashion explained earlier in the thesis, as the aim was to identify a strength of relationship, direction and significance levels when data were regressed against fish age, length and mass, and not to determine the exponent of the allometric relationship associated with the scaling.

Results

It appears that the most prominent difference in how age/size affected activity of enzymes between snapper and YEM was that, generally, snapper exhibited predominantly negative relationships while YEM were inclined to have more positive responses as they grew (Table A2.1). YEM generally possess greater aerobic capacity compared to snapper, which at least partially could be explained by the fact that activities of aerobic energy generation increased with age/size. Overall, the least affected tissue, in this respect, was found to be red muscle, where in many instances levels of enzymatic activity, per gram of wet tissue mass, was preserved (unchanged) irrespective of fish size/age.

Table A2.1. Descriptive statistics (i.e. coefficient of correlation R, coefficient of determination R^2, F statistics, p-values and type – positive POS or negative NEG direction of the relationship) describing relationships between snapper and YEM age, body length and mass with activities of aerobic (CCO – cytochrome c oxidase, CS – citrate synthase, HOAD – 3-hydroxyacyl-CoA dehydrogenase) and glycolytic enzymes (LDH – lactate dehydrogenase and PK – pyruvate kinase) in selected tissues (WM – white muscle, RM – red muscle, INT – intestines and liver) determined for the period of one calendar year under unlimited food regime. Where results were not significant at $\alpha \leq 0.05$, statistical information was not provided and is indicated with N/P (not applicable).

SNAPPER

	AGE					LENGTH					MASS				
ENZYMES	R	R^2	F STATS	P-VALUE	TYPE	R	R^2	F STATS	P-VALUE	TYPE	R	R^2	F STATS	P-VALUE	TYPE
CCO															
WM	0.33	0.11	18.58	< 0.001	NEG	0.52	0.27	55.01	< 0.001	NEG	0.55	0.31	62.94	< 0.001	NEG
RM	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P
INT	0.35	0.12	20.38	< 0.001	NEG	0.51	0.25	45.57	< 0.001	NEG	0.44	0.19	35.21	< 0.001	NEG
CS															
WM	0.36	0.13	22.01	< 0.001	NEG	0.23	0.05	8.28	0.005	NEG	0.23	0.06	8.63	0.004	NEG
RM	0.43	0.19	32.03	< 0.001	NEG	0.49	0.24	45.67	< 0.001	NEG	0.44	0.19	34.01	< 0.001	NEG
INT	0.44	0.21	35.71	< 0.001	NEG	0.48	0.23	42.75	< 0.001	NEG	0.43	0.18	33.18	< 0.001	NEG
HOAD															
WM	0.25	0.06	9.82	0.002	NEG	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P
RM	0.31	0.09	14.71	< 0.001	POS	0.47	0.22	40.27	< 0.001	POS	0.45	0.21	37.71	< 0.001	POS
LIVER	0.18	0.03	4.78	0.003	NEG	0.28	0.08	11.98	< 0.001	NEG	0.23	0.05	7.81	0.006	NEG
LDH															
WM	N/P	N/P	N/P	N/P	N/P	0.46	0.21	38.79	< 0.001	POS	0.46	0.21	39.58	< 0.001	POS
RM	0.35	0.12	22.11	< 0.001	NEG	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P
PK															
WM	0.61	0.36	82.97	< 0.001	POS	0.57	0.32	70.93	< 0.001	POS	0.54	0.29	59.51	< 0.001	POS
RM	0.35	0.12	20.26	< 0.001	NEG	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P

YEM

ENZYMES	**AGE**					**LENGTH**					**MASS**				
	R	R^2	F STATS	P-VALUE	TYPE	R	R^2	F STATS	P-VALUE	TYPE	R	R^2	F STATS	P-VALUE	TYPE
CCO															
WM	0.36	0.13	29.42	< 0.001	POS	0.31	0.09	20.88	< 0.001	POS	0.28	0.08	17.02	< 0.001	POS
RM	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P
INT	0.49	0.24	64.07	< 0.001	POS	0.42	0.18	44.61	< 0.001	POS	0.39	0.15	36.61	< 0.001	POS
CS															
WM	0.62	0.39	129.59	< 0.001	POS	0.56	0.31	93.89	< 0.001	POS	0.51	0.25	70.37	< 0.001	POS
RM	0.31	0.09	21.48	< 0.001	POS	0.26	0.07	14.71	< 0.001	POS	0.21	0.04	8.34	0.004	POS
INT	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P
HOAD															
WM	0.23	0.05	11.82	< 0.001	NEG	0.24	0.06	12.89	< 0.001	NEG	0.27	0.07	16.28	< 0.001	NEG
RM	0.21	0.04	9.08	0.003	POS	0.16	0.02	5.27	0.023	POS	N/P	N/P	N/P	N/P	N/P
LIVER	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P
LDH															
WM	0.45	0.21	52.95	< 0.001	POS	0.47	0.22	57.84	< 0.001	POS	0.42	0.18	44.09	< 0.001	POS
RM	0.15	0.03	5.03	0.003	NEG	0.24	0.06	12.32	< 0.001	NEG	0.24	0.06	12.58	< 0.001	NEG
PK															
WM	0.52	0.27	76.06	< 0.001	POS	0.51	0.25	69.91	< 0.001	POS	0.45	0.21	52.31	< 0.001	POS
RM	N/P	N/P	N/P	N/P	N/P	0.16	0.03	5.43	0.021	NEG	0.19	0.04	7.88	0.005	NEG

APPENDIX 3

Incorporation of data from Coxon (2014) with the present study snapper data

Introduction

Coxon (2014) was a study focused on the exercise physiology of snapper with implications for improved commercial harvesting. Specimens for her work were obtained from the same snapper hatchery/rearing facilities (i.e. PFR, the seafood unit Nelson) as for the present study, therefore genetic and phenotypic characteristics of test fish between the two studies were practically identical. In addition, Coxon (2014) measured resting (routine, RMR) and maximum metabolic rates (MMR) to produce aerobic scope using the same equipment and protocol on fish of comparable size to ones used in the present study, but at different temperatures (i.e. 12, 18 and 24°C, and 13, 17 and 21°C in Coxon, 2014, and the present study respectively). This allowed for incorporation of data generated in Coxon (2014) wherever appropriate, to strengthen certain arguments and suggestions raised in the present study by increasing a power of statistical analysis via increasing a sample size. This approach was applicable in chapters 3 and 7.

Incorporation of Coxon (2014) data to chapter 3

In chapter 3, data from Coxon (2014) were suitable to be combined with the present study data in the two scenarios.

Appendix 3.1 First scenario

In the present study snapper RMR did not exhibit a typical teleost exponential increase as a function of temperature (Jobling, 1994). However, when three additional RMR data points from Coxon (2014)

obtained at different temperatures; were combined with the existing data, the relationship between the resting metabolism and ambient temperature did show an overall exponential character (Fig. A3.1). For a more detailed explanation refer to the discussion section in chapter 3.

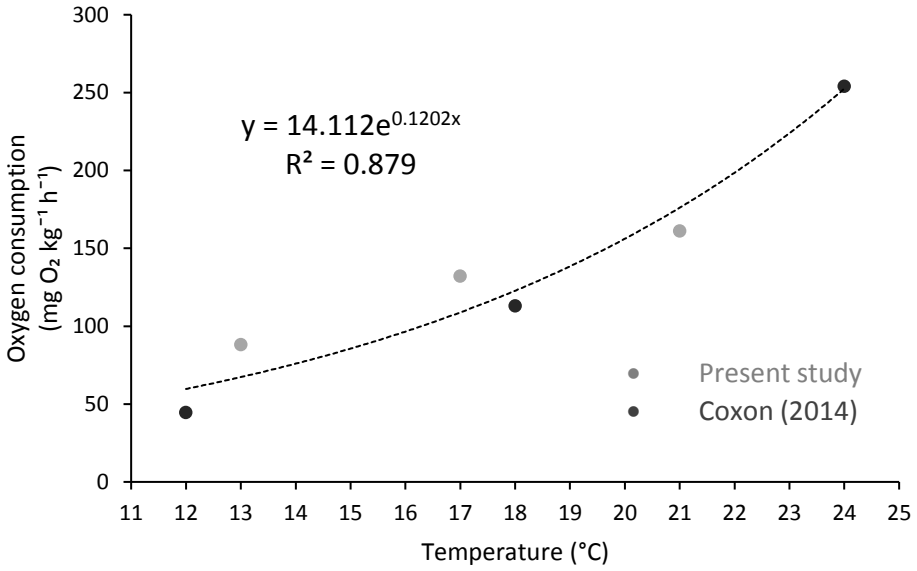

Figure A3.1. Relationship between resting (routine) metabolic rates and temperature with combined snapper data from the present study (light grey symbols) and Coxon (2014) (dark grey symbols) with the curve representing the exponential model explaining the data with associated equation and the coefficient of determination (R^2).

Appendix 3.2 Second scenario

Body mass is one of the most significant determinants of metabolic rate. It is well established that scaling of metabolism with body mass can be expressed with the power function $y = aM^b$, where the most important component of the equation is exponent *b* reflecting the extent to which an increase in mass affects an increase in metabolic rates (Brett and Groves, 1979; Bokma, 2004; White et al., 2006; Killen et al., 2007). Coxon (2014) reported this relationship for snapper in both forms, when metabolic rates were expressed either as per gram of wet body mass (i.e. mass-specific oxygen consumption, MO_2) or per total fish mass (i.e. absolute MO_2) with the associated allometric equations and *b* exponents. These data were used to evaluate the effects of lower environmental temperatures on activities and metabolism of snapper and assisted in providing further support to a notion that

snapper, similar to *Sparus aurata* (a Mediterranean cousin), are sensitive to a drop in ambient temperature below 13°C (Ibarz et al., 2010).

Mass-specific and absolute MO_2 data at 12°C for two different mean snapper masses from Coxon (2014) were grouped with the same type of data at 13°C from the present work but with a different mean mass to the two means from Coxon (2014). This allowed construction of allometric relationships for the lower temperature range. The same was carried out for the 17°C (this study) and 18°C (Coxon, 2014), thus observational comparisons between higher and lower temperature ranges could be performed. The same procedure was repeated for both RMR (Fig. A3.2) and MMR (Fig. A3.3), with the intention to investigate if there was similarity in how allometric relationships were affected by the different temperatures (i.e. middle range temperature 17/18°C and lower range temperature 12/13°C that snapper experience in the wild) between resting and maximum MO_2. Graphical expressions of the combined data, as illustrated in Fig. A3.2 and A3.3, pointed out a much better fit at the higher temperature range for both mass-specific (Fig. A3.2B and A3.3B) and absolute MO_2 (Fig. A3.2D and A3.3D) than for combined data at the lower range, which was particularly obvious for RMR MO_2 (Fig. A3.2). Even though scaling exponent *b* appeared to be similar with what was reported in Coxon (2014) at both temperature levels for RMR (i.e. -0.26 and 0.74 for routine mass-specific and absolute MO_2 respectively in Coxon, 2014; -0.28 and 0.72 for routine mass-specific and absolute MO_2 in combined data at 17/18°C respectively; and -0.26 and 0.74 for routine mass-specific and absolute MO_2 in combined data at 12/13°C respectively), it was obvious that at the lower temperature range the model poorly represented the data which was due to much higher values observed for 13 than 12°C. In the case of MMR, at the lower temperature range even the exponent *b* was distinctly different from the values observed in Coxon (2014) (i.e. -0.19 and 0.81 for maximum mass-specific and absolute MO_2 respectively in Coxon, 2014; -0.13 and 0.87 for maximum mass-specific and absolute MO_2 in combined data at 17/18°C respectively; and 0.04 and 1.04 for maximum mass-specific and absolute MO_2 in combined data at 12/13°C respectively), indicating that differences between 17 and 18°C may not have any marked impact on metabolic expenditure of this species. However, quite the opposite was observed at the lower temperature range. Metabolic activity at 13/12°C for both RMR and MMR, demonstrated that the step from 13°C down to 12°C may be an important metabolic shift for snapper at which point a clear metabolic reduction was observed. For further explanation of the phenomenon refer to discussion in chapter 3.

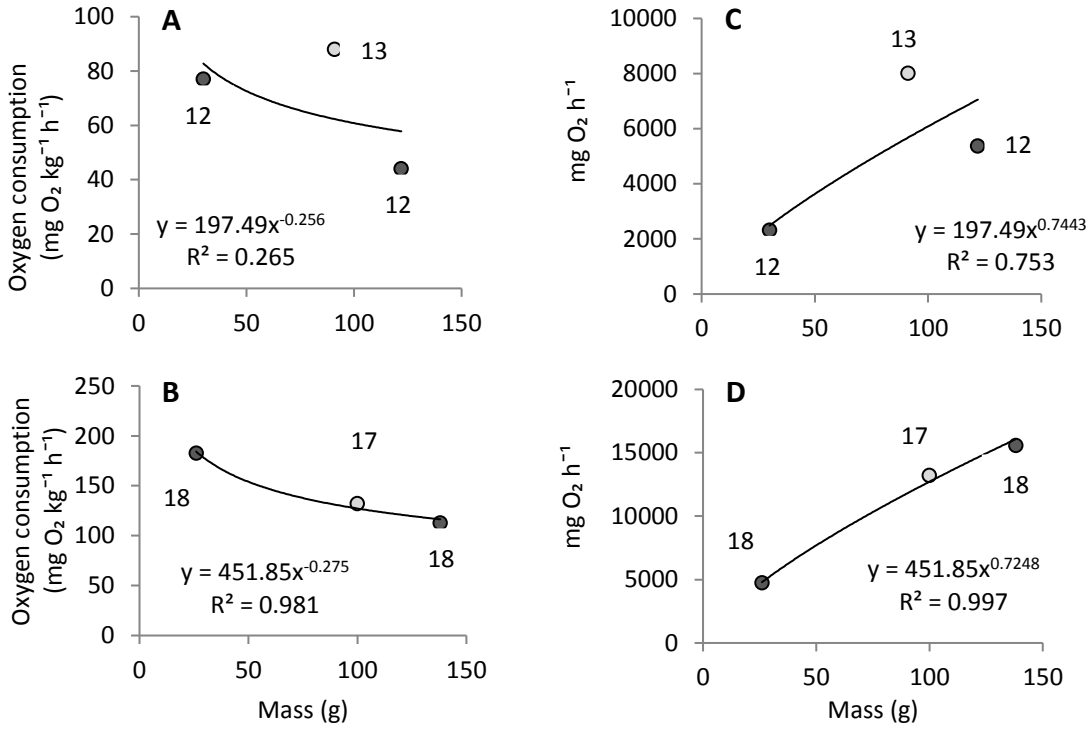

Figure A3.2. Allometric changes in mass-specific (A and B) and absolute (C and D) routine oxygen consumption with snapper mass. Light grey symbols represent data from the present study and dark grey from Coxon (2014); associated numbers are temperatures at which oxygen consumption was measured in the two studies. Equations with R^2 represent the power function used to fit the data with *b* of the relationship in the exponent in the model.

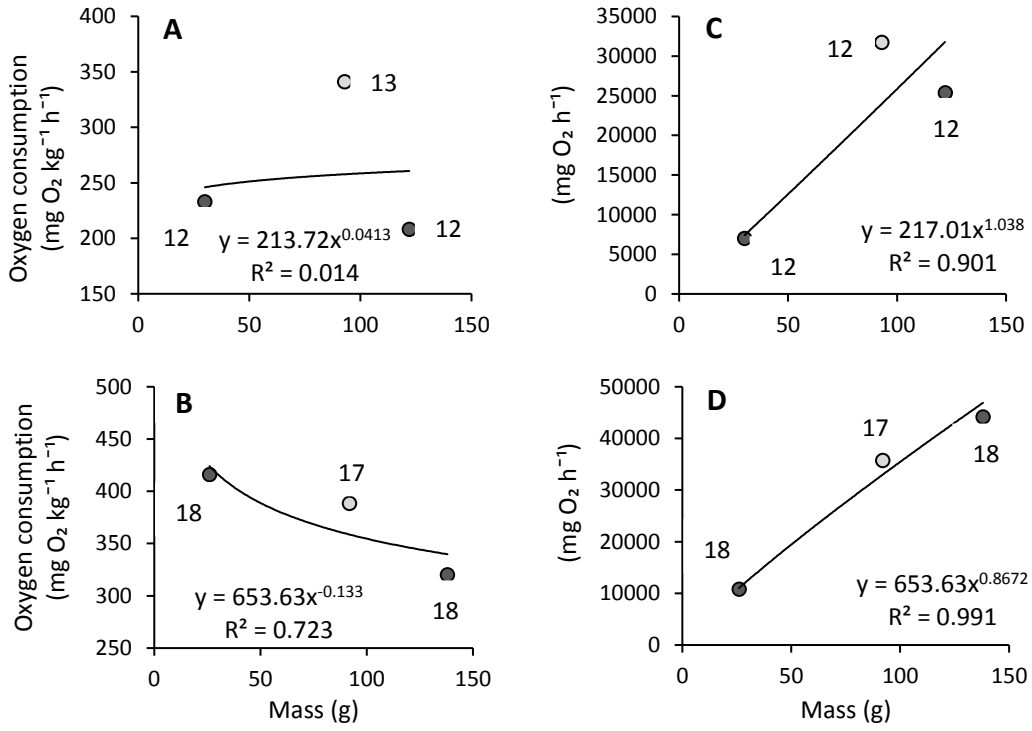

Figure A3.3. Allometric changes in mass-specific (A and B) and absolute (C and D) maximum oxygen consumption with snapper mass. The rest of the figure caption is the same as in Fig. A3.2.

Appendix 3.3 Incorporation of Coxon (2014) data to chapter 7

The link between metabolic rates (RMR and MMR) and associated aerobic metabolic scope (AS) with growth rates (GR) in fish have been a focus in many investigations (e.g. Auer et al., 2015a; 2015b; Rosenfeld et al., 2014; Biro and Stamps, 2010; Burton et al., 2011). In the present study this was also possible; however, available data could only produce three sample pairs for three testing temperatures (i.e. 13, 17 and 21 °C, light grey symbols in Fig. A3.4) which made testing with the general linear model less reliable. Adding another three data pairs originating from Coxon (2014) (i.e. 12, 18 and 24 °C, dark grey symbols in Fig. A3.4) markedly increased statistical power so that certain correlations could be statistically supported (Fig. A3.4A, B, D and E) while others could not (Fig. A3.4C and F) (for details about the observed patterns refer to discussion in chapter 7).

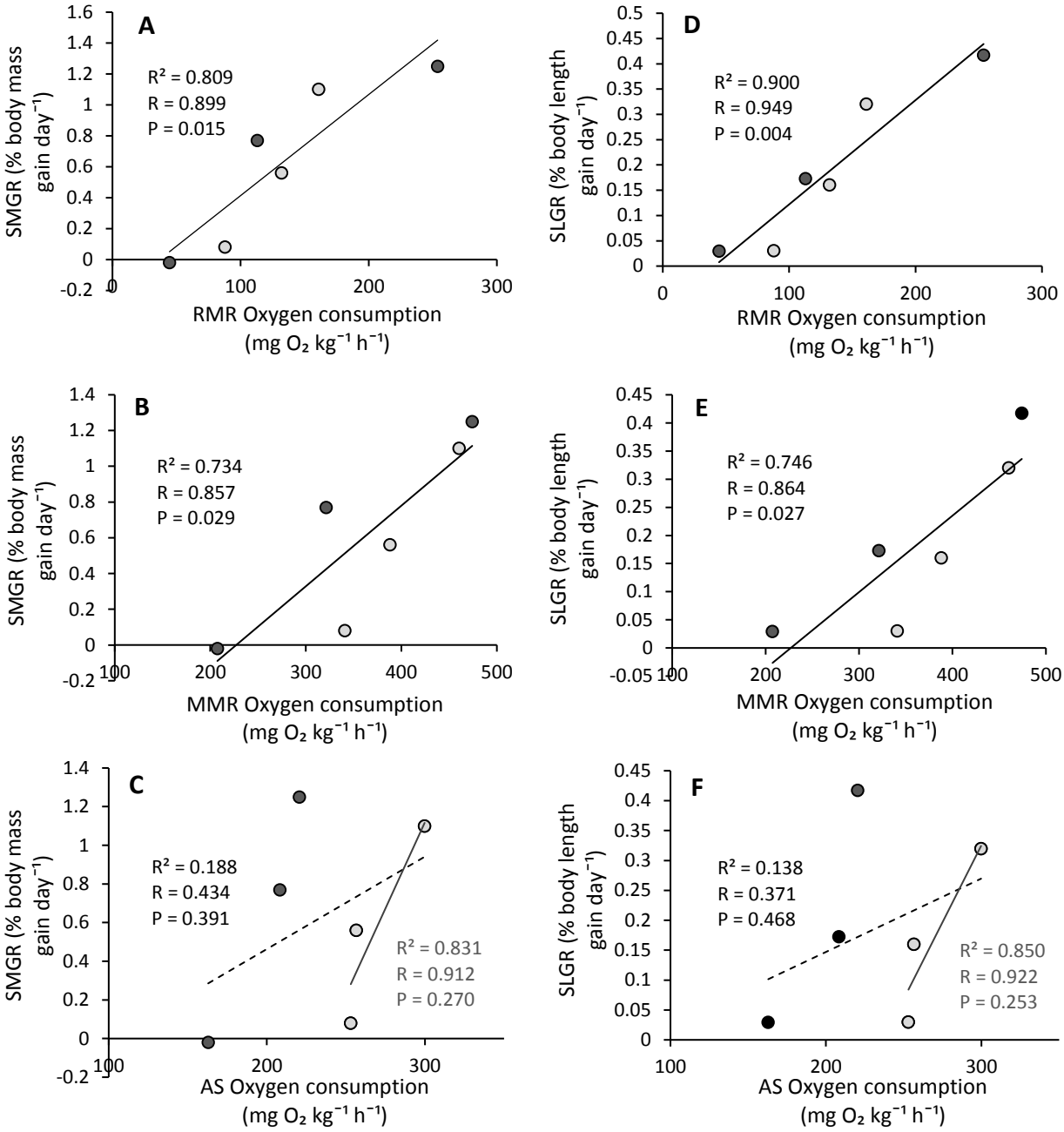

Figure A3.4. Specific mass (SMGR, A–C) and length (SLGR, D–F) growth rates of snapper as a function of routine metabolic rate (RMR) (A, D), maximum metabolic rate (MMR) (B, E) and absolute aerobic scope (AS) (C, F). Light grey symbols represent data from the present study and dark grey from Coxon (2014). Black full and dashed lines represent significant and non-significant linear regression lines respectively, with coefficients of determination (R^2), correlation coefficient (R) and p-values for the combined data. In C and F, grey dashed lines, R^2s, Rs and p-values belong to data from the present study only.

APPENDIX 4

Metabolic phenotype and growth performance in YEM

Association between metabolic phenotype and growth was also evaluated for YEM. Similar to snapper (Appendix 3.3), correlation between the two variables were assessed when YEM metabolic data generated for three testing temperatures were paired with corresponding GR data from the feeding experiment (Fig. A4.1). A low sample size did not allow detection of significant correlations even when this looked plausible (e.g. Fig. A4.1D). Nevertheless, for the majority of correlations, particularly those associated with SMGR, no link between metabolic activity and growth performance was observed, therefore no suggestion about an association between growth and metabolic activity could be made based on data from the present work.

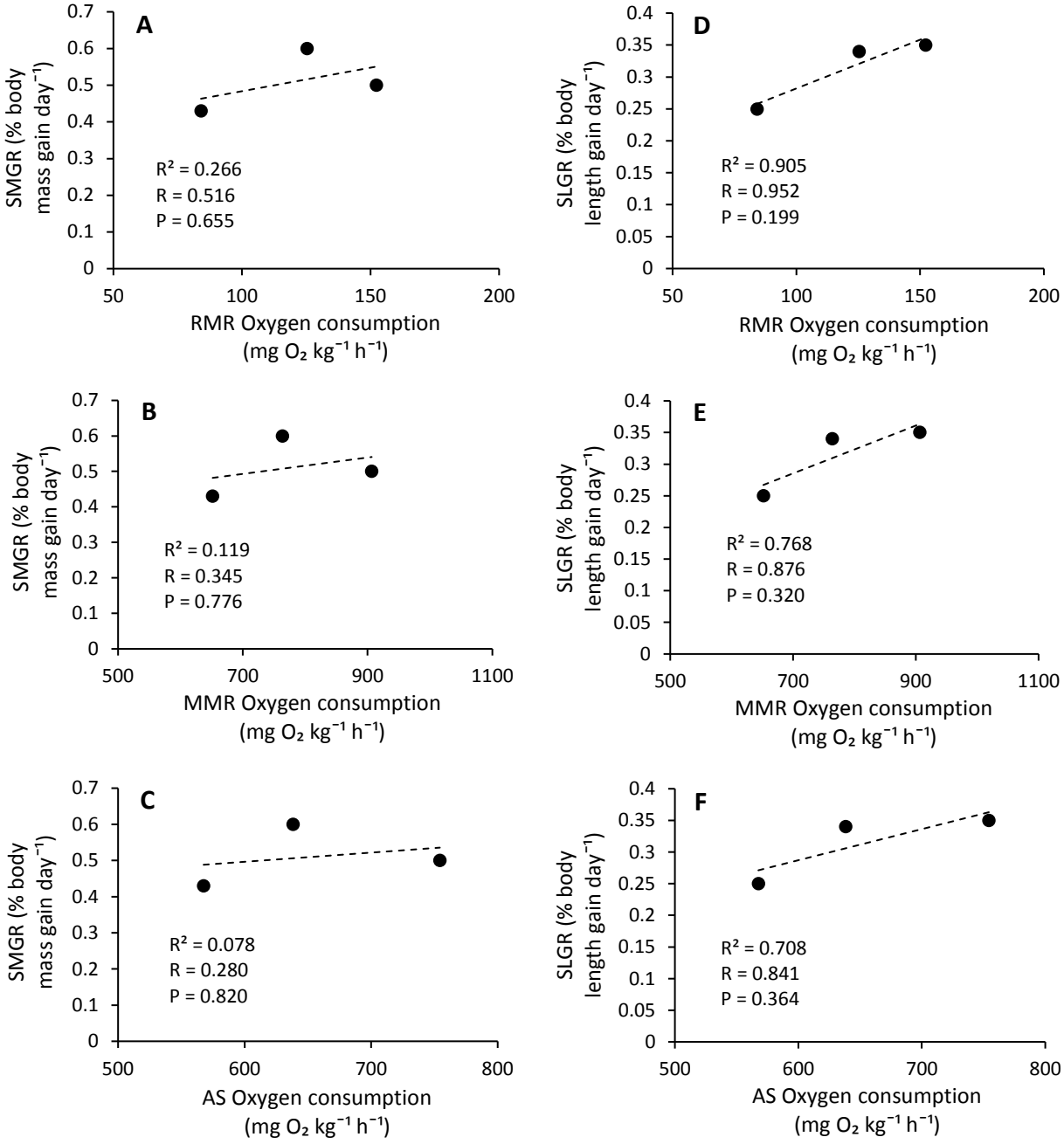

Figure A4.1. Specific mass (SMGR, A–C) and length (SLGR, D–F) growth rates of YEM as a function of routine metabolic rate (RMR) (A, D), maximum metabolic rate (MMR) (B, E) and absolute aerobic scope (AS) (C, F). Black symbols represent data and dashed lines non-significant linear regression lines with coefficients of determination (R^2), correlation coefficient (R) and p-values.

APPENDIX 5

Specific dynamic action and growth performance in snapper

Specific dynamic action (SDA) is an increase in metabolic rate due to meal ingestion. How quickly this response lasts (i.e. time needed for post-prandial MO_2 to return to the levels of pre-prandial rates) and what the dynamics of it are, or in other words how quickly, high and how much of MO_2 in total is devoted to processing a meal, may determine the rate an organism will grow. Therefore, a strategy based on rapid digestion may support a higher frequency of feeding, which could mean that greater amounts of nutrients are assimilated to the body per unit time, which would ultimately increase GR (Metcalfe et al., 2016). However, amongst a range of fish species where the relationship between SDA response and GR was tested, different patterns, to support all three possible outcomes, have been observed: that is, SDA was found to positively, negatively or not relate to growth performance. To test where on this gradient the test species were, it was, in the present study, partially permissible only for snapper (for details see discussion in chapter 4).

Data were obtained as described in chapter 2 (GR) and 4 (SDA). Corresponding GR data at matching temperatures were paired with SDA data measured at 13, 17 and 21°C. The statistical approach used was based on the general linear model, in a way as explained earlier, to generate coefficients of determination (R^2) and associated p-values for three SDA parameters (SDA peak, cost and duration) as a function of SMGR and SLGR (Fig. A5.1). The size of an ingested meal in unlimited food trials may not match with the set ration that fish in SDA trials were fed with, thus the true values of SDA responses at equivalent temperatures were likely not accurately estimated. Nevertheless, the relationships were characterised based on available data (Fig. A5.1) and interpreted in the light of an assumption that actual rations ingested during the tank array feeding trials would not change the direction (i.e. positive/negative) of associations between SDA parameters and GR, as described in detail in chapter 7. According to results portrayed in the Fig. A5.1, in chapter 7, a suggestion was made that in snapper, SDA may have had positive effects on growth, since SDA parameters, that have been demonstrated to be associated with growth promotion, showed a type of relationship that was aligned with the suggestion (i.e. SDA peak and cost were positively and SDA duration negatively correlated with GR).

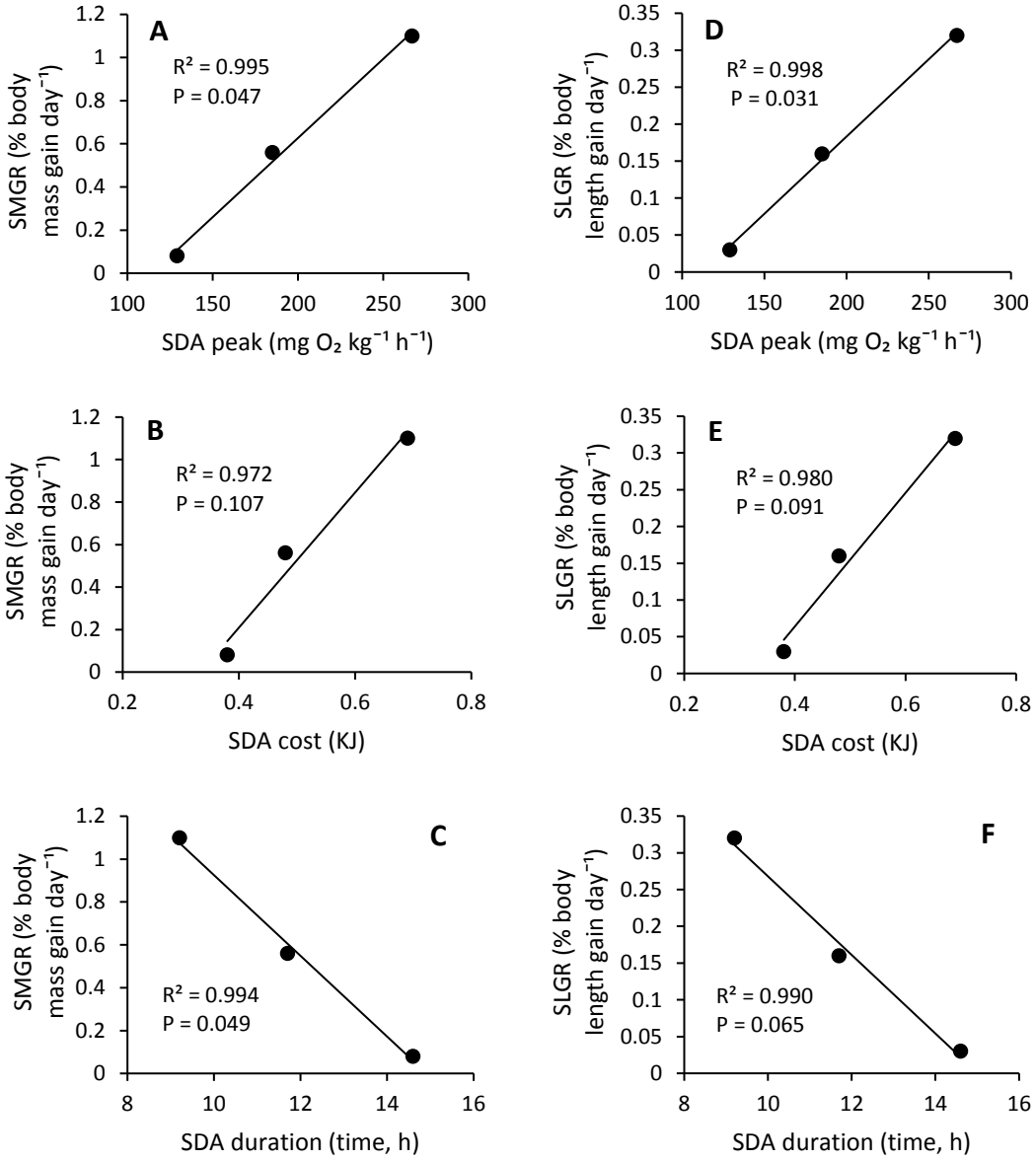

Figure A5.1. Specific mass (SMGR, A–C) and length (SLGR, D–F) growth rates as a function of SAD peak (A, D), SDA cost (B, E) and SDA duration (C, F). Black symbols and lines represent data and linear regression lines respectively with associated coefficients of determinations (R^2) and p-values.